The Survival of Red Halophiles in Water and in Brines

By C. H. Castell and E. G. Mapplebeck
Atlantic Fisheries Experimental Station, Halifax, N.S.
(*Received for publication December 2, 1951*)

ABSTRACT

The survival time of red halophiles in solutions of sodium chloride is greatest in saturated solutions and decreases with reductions in the concentration of salt. At 25°C. heavy inoculations may survive for months in saturated brines, rarely over 24 hours in 10 per cent solutions, and for 2 to 6 hours in tap water free from salt. Increased temperature and decreased pH reduced the survival time in all of the salt concentrations tested. Various protein materials, but not sugar, had a protective action against the destructive action of decreased salt concentrations.

Under ordinary conditions the red bacteria found in solar salt are considered to be absolute halophiles. Lochhead (1934), working with four different species, found the growth range to lie between 16 and 32 per cent sodium chloride. Gibbons (1936) found the range for several species to lie between 10 and 35 per cent with only meagre growth between 10 and 15 per cent.

Stuart and James (1938) observed that transfers made from very old cultures were capable of growing in media with lower salt content, and Hess (1942) has shown that the range of salt tolerance for these organisms can be extended in both directions by gradual adaptation, but none could be adapted to less than 6 per cent and 18 out of 25 cultures could not be adapted to less than 12 per cent salt.

Stuart (1940) has shown that the effect of pH, protein concentration and sodium chloride concentration on the growth of halophiles are to some extent dependent upon each other.

These and other similar studies refer to the concentration of sodium chloride necessary for continued growth in an otherwise favourable substrate. When the salt concentration is lowered to the point where the organisms are no longer able to proliferate, it does not necessarily follow that the cells will die immediately.

The work described in this paper was done chiefly to determine the effect of the salt concentration on the survival of red halophiles in brines. Because of the rapidity at which these organisms die in solutions free from sodium chloride, an attempt was also made to compare the sterilizing action of ordinary tap water with that of some common disinfectants, for these particular bacteria.

EXPERIMENTAL METHODS

Organisms

The cultures used were isolated from salt fish and solar salt. One group consisted of gram-negative rods identified as being *Pseudomonas salinaria* Harrison and Kennedy. These were the types most frequently found on the discoloured salt

J. Fish. Res. Bd. Can., 9 (8), 1952.
Printed in Canada.

cod. The majority of the tests were made with two strains, #18 and #2. Less frequently found, but also present on spoiled salt cod and in solar salt were cocci identified as *Sarcina littoralis* Poulsen.

Culture Medium

The culture medium used was a modification of Lochhead's (1934) skim milk salt agar, consisting of the following:

250 g. sodium chloride
50 g. calcium carbonate
50 g. Klim (powdered milk)
5 g. calcium nitrate
10 g. Bacto Loefflers blood serum
15 g. agar
1000 ml. water

The agar was dissolved in the water by boiling. The other ingredients were then stirred into the hot agar solution and thoroughly mixed in a preheated Waring blender. As autoclaving tended to produce a lumpy medium, it was sterilized by 10 minutes boiling. (Heat resistant, spore-forming halophiles were never encountered.) After sterilization the medium was poured directly into sterile Petri plates and allowed to harden.

Dilution blanks were prepared by autoclaving 16 per cent saline.

The terms salt or sodium chloride refer to C. P. sodium chloride; the terms percentage or per cent have been used to indicate the number of grams of salt added to 100 ml. of liquid.

Procedures Used

Two types of test were made. The first was used to determine the survival period of the red halophiles in water and various brine solutions. This consisted of inoculating one-liter amounts of the brines or water with one ml. of a saline suspension of an actively growing culture containing over a billion cells per ml. Periodically, representative one ml. samples were withdrawn from each flask and transferred to the surface of sterile salt agar medium in Petri plates. These were incubated at 42°C. and a record kept, not only where growth occurred, but also of the time that elapsed before growth became visible on the plates.

The second type of test was used to evaluate water and other solutions as sterilizing agents for the red halophiles.

For this purpose, brine-soaked pieces of unpainted wood, having a uniform size ($11 \times 1 \times 0.2$ cm.) and surface texture were immersed for 30 minutes in a very heavy suspension of red halophiles in 16 per cent brine. These contaminated pieces of wood were then submerged for measured periods of time in the water or the disinfectant solution being tested. So as to have an excess of the disinfectant, the weight of the solution to the weight of the wood in every case was approximately 750:1.

To measure the survival of the organisms on the treated wood, the following method was used:

Each piece was quickly shaken to remove excess moisture and then immersed into 1000 ml. of 16 per cent sterile brine and shaken vigorously. Five serial dilutions were made from this brine and 5 ml. transferred from each to the surface of the salt agar medium. As in the previous tests, these were incubated at 42°C. and examined daily for the development of red growth.

Control checks in which similarly contaminated pieces of wood were shaken in the sterile 16 per cent saline were used in every set of tests to give an approximate idea of the number of red organisms on the wood before treatment.

EXPERIMENTAL RESULTS

SURVIVAL IN BRINES. In the first set of tests the brines were stored at room temperature (18° to 25°), and away from direct sunlight. Under these conditions, in four sets of tests, none of the cultures survived for 24 hours in brines containing 1 or 5 per cent salt. One of the four survived for 24 but not 42 hours, in 10 per cent salt. In 15 per cent they lived from 20 to 41 days. In 20 per cent, none survived beyond 76 days and in saturated solutions, none survived beyond 120 days.

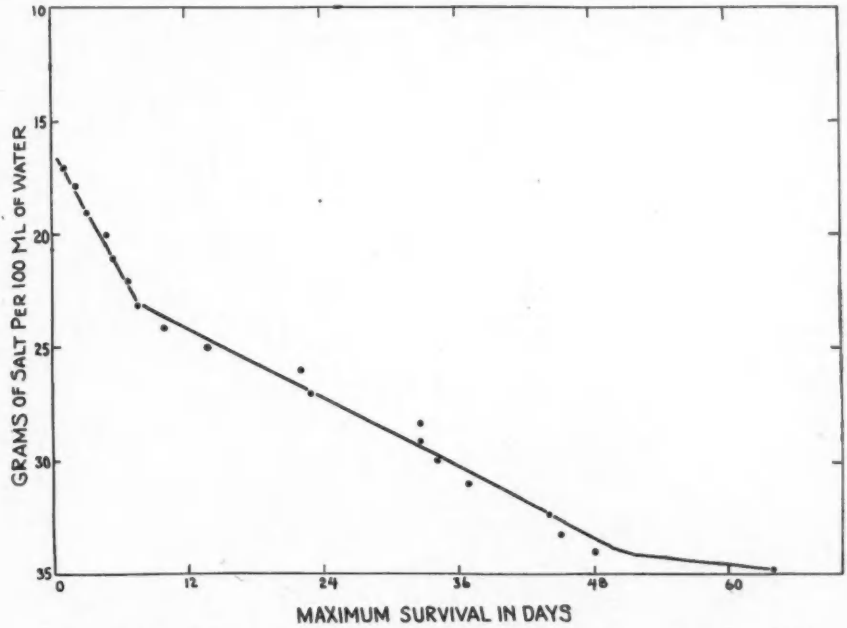


FIGURE 1. The effect of salt concentration on the survival of red halophiles (*Ps. salinaria* #5) in brines stored at 22° to 25°C.

Figure 1 gives the results of one of these tests in greater detail, showing that as the concentration of salt is increased, there is a corresponding increase in the period of survival.

EFFECT OF TEMPERATURE ON SURVIVAL IN BRINES. There is definitely no relationship between the so-called optimum growth temperature (37° to 43°) and the optimum temperature for the survival of these organisms in brines (Table I). Even in solutions containing 20 per cent salt, the cells did not survive for 24 hours at 45°. As the temperature is reduced the survival is increased for each level of salt concentration. Below 0° they survive for several days in 5 per cent brines.

TABLE I. The maximum survival time, in days, for suspensions of *Ps. salinaria* in solutions of sodium chloride stored at various temperatures.

Salt conc.	Storage temperatures							
% W/v	− 23°	− 0.5°	3°	7.5°	25°	37°	40°	45°
5	15	5	0	0	0	0	0	0
10	21	1	5	4	1	0	0	0
15	86*	86	86	86	20	0	0	0
20	86	86	86	86	86	16	44	0

*Experiment terminated at 86 days.

TABLE II. The effect of adding 1 per cent blood serum to the survival time of cells of *Ps. salinaria* in brine solutions.

Salt conc.	Days survival	
% W/v	Control	+ Blood serum
1	0	0
5	0	2
10	0	89
15	20	130
20	66	145

TABLE III. The maximum survival time, in days, for suspensions of *Ps. salinaria* in solutions of sodium chloride containing from 0 to 5 per cent gelatin at 22°C.

NaCl	Per cent gelatin				
%	0	0.01	0.1	1	5
0	0	0	0	0	0
1	0	0	0	0	0
5	0	0	0	0	0
10	0	0	6	> 70	> 70
15	41	44	50	> 70	> 70
20	> 70	64	> 70	> 70	> 70

It is interesting to compare this with the effect of dry heat on these organisms in contaminated salt. It was observed for example (Castell, 1950) that after 26 hours of continuous dry heat at 52°C., the organisms remained viable, growing rapidly on being transferred to a suitable medium.

EFFECT OF ADDED PROTEINS. One per cent dextrose added to the brines made no significant change in the survival times. One per cent of various proteins had a marked protective action (Tables II and III). Cells which were destroyed within 24 hours in solutions containing 10 per cent salt, survived for 89 days in similar solutions to which one per cent blood serum had been added. Peptone had a similar action, but with this difference—there was always an increased lag in the subsequent growth of the cultures that had been in brines containing peptone. The results obtained by using different amounts of gelatin would suggest a definite relationship between this protective action and the concentration of the protein (Table III).

WATER AS A DISINFECTANT

In the preceding experiments, except at very low temperatures, heavy suspensions of *Ps. salinaria* did not survive for 24 hours in brines having less than 5 per cent salt. How long they survived at salt concentrations between 0 and 5 per cent was not determined. In the next series of tests an effort was made to determine the survival time of cells of *Ps. salinaria* and *Sar. littoralis* on the surface of contaminated wood, using plain tap water, and then to compare this with the results of similar tests using some common disinfectants.

TABLE IV. The destruction of red halophilic bacteria on pieces of wood immersed in tap water for various periods of time, as indicated by the number of living cells that could be washed off the wood surface after treatment. (+ or — indicates growth or no growth on salt medium. The figures in brackets indicate the number of days that elapsed before visible growth occurred on the plates.)

Hours in tap water	Growth from dilutions of the saline rinse									approx. % decrease
	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Control	+ (3)	+ (4)	+ (4)	+ (5)	+ (5)	+ (6)	+ (8)	+ (7)	—	0
0.5 hours	+ (4)	+ (5)	+ (5)	+ (6)	+ (6)	+ (7)	—	—	—	99
1 "	+ (5)	+ (5)	+ (6)	+ (7)	+ (7)	+ (9)	—	—	—	99
2 "	+ (5)	+ (6)	+ (7)	+ (10)	+ (10)	—	—	—	—	99.9
3 "	+ (6)	+ (7)	+ (10)	+ (11)	—	—	—	—	—	99.99
4 "	+ (18)	+ (12)	+ (15)	—	—	—	—	—	—	99.999
5 "	+ (11)	+ (32)	+ (39)	—	—	—	—	—	—	99.999
6 "	+ —	—	—	—	—	—	—	—	—	100

Table IV gives the results of a typical experiment. The organisms used were *Ps. salinaria* #18 and there were approximately 10 million cells per ml. in the rinse water of the control test. It can be seen that even one hour in the tap water reduced the number of live cells by approximately 99 per cent. And each succeeding hour brought a further decrease. It can also be seen that the relatively few cells that did survive five hours in tap water required a very much longer time

to re-establish growth on the salt agar medium. In a similar set of nine tests, using tap water as the disinfectant, the maximum survival times were as follows:

Ps. salinaria #18 between 5 and 6 hours
Ps. salinaria #18 between 4 and 5 hours
Ps. salinaria #18 between 5 and 6 hours
Ps. salinaria #18 between 2 and 3 hours
Ps. salinaria # 2 between 1 and 2 hours
Ps. salinaria # 2 between 1 and 2 hours
Ps. salinaria # 2 between 2 and 3 hours
Ps. salinaria # 2 between 1 and 2 hours
Sar. littoralis # 1 between 2 and 3 hours

By replacing tap water (pH 5.1) with distilled water there was a very slight increase in the rate at which the cells were destroyed in some, but not all of the tests. Sea water, on the other hand, was much slower in destroying the cells. In tests that were set up together, the cells survived four to six times as long in sea water as they did in tap water. In none of the tests were these halophiles found to survive for 24 hours or more in sea water at room temperature (25°C.).

EFFECT OF TEMPERATURE ON THE STERILIZING ACTION OF WATER. The lethal effect of water on the red halophiles increased as the temperature was raised. Ordinary tap water at 70°C. destroyed all the red organisms on heavily contaminated wood in less than five minutes. Water at 70°C. is a better disinfectant than one per cent hydrochloric acid, two per cent lye, 1000 p.p.m. chlorine or 5 per cent formaldehyde at 20° to 25°C.

Figure 2 shows the effect of temperatures between 22° and 70° on the survival of *Ps. salinaria* on contaminated wood submerged in tap water. This represents one of a series of similar tests, including some with *Sar. littoralis*, all of which gave approximately the same results. The range of time for the complete destruction of heavy inoculations of all the strains at the different temperatures are as follows:

25° 1 to 6 hours
38° 40 to 75 minutes
48° 35 to 40 minutes
55° 12 to 20 minutes
70° 2 to 5 minutes
100° less than 1 minute

EFFECT OF pH. Buffer solutions were made up using 0.2 Na_2HPO_4 with 0.1 M citric acid, and 0.1 M NaOH with sodium citrate to give a pH range between 3.4 and 11.2. Five hundred ml. of each of these solutions were diluted eight times in water and then used as disinfecting solutions for the pieces of wood contaminated with *Ps. salinaria* #2. The results (Table IV) show that as the pH decreases the cells are destroyed more rapidly.

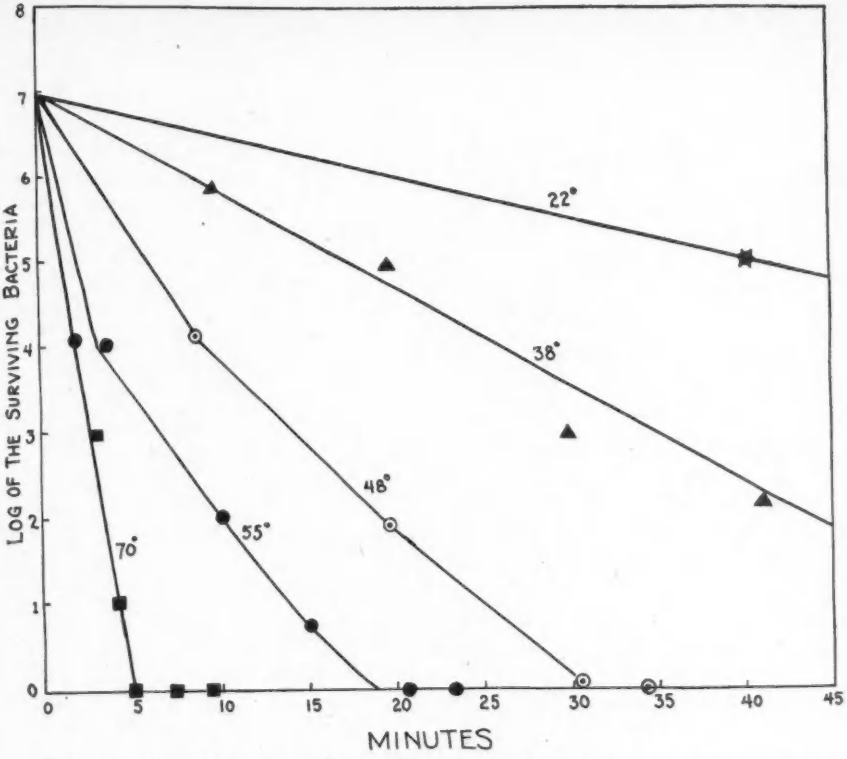


FIGURE 2. The effect of temperature on the survival of red halophiles on wood immersed in tap water (*Ps. salinaria*).

COMMON DISINFECTANTS

In order to get data comparable to the results obtained with water, over 150 similar sets of tests were made by immersing similarly contaminated pieces of wood in solutions of various disinfectants for 2 to 30 minutes. A brief summary of these results follows:

(1) QUATERNARY AMMONIUM COMPOUNDS. Two commercial products, "Roccal" and "Amosol", were used. The former in concentrations up to 3 per cent and the latter up to 1 per cent, for exposures up to 30 minutes were wholly ineffective. It did not seem worth while to run further tests with higher concentrations.

(2) FORMALDEHYDE. Solutions containing from 0.1 to 5 per cent formalin (40 per cent formaldehyde) with exposures up to 30 minutes were wholly ineffective, Although very high concentrations (i.e. 50 ml. of formalin in 1000 ml.) did not destroy the cells after 30 minutes, it did affect the cells so that they

TABLE V. The destruction of red halophiles on wood by immersion in weak buffer solutions with pH varying between 3.4 and 11.2.

pH	Buffer	Time for complete destruction
3.4	Na_2HPO_4—citric acid	between 1 and 2 hours
4.4	" " "	" 1 " 2 "
6.2	" " "	" 2 " 3 "
7.1	" " "	" 2 " 3 "
8.0	" " "	" 2 " 3 "
8.0	NaOH—Na citrate	" 2 " 3 "
11.0	" " "	" 3 " 4 "
5.2	Tap water control	" 1 " 2 "

required a period of 9 to 16 days to re-establish growth on being transferred, compared to 3 to 7 days for the control. Also, very frequently the transfers made directly from the saline in which the treated wood was rinsed failed to produce growth, while transfers made from dilutions of this saline produced abundant growth, suggesting a bacteriostatic action.

(3) COPPER SULPHATE. From 0.1 to 1 per cent copper sulphate for 30 minutes was ineffective with cultures of *Ps. salinaria*; 1 per cent for 30 minutes, but not 10 minutes, destroyed almost all the cells of *Sar. littoralis*.

(4) CHLORINE. These solutions were made from "Oakite", a commercial chlorine product. Up to 0.1 per cent available chlorine with exposures up to 10 minutes was ineffective; 0.1 per cent for 15 to 30 minutes caused a considerable reduction; and 0.2 per cent for 15 minutes completely destroyed all the organisms.

(5) LYE (sodium hydroxide). Two per cent with exposures up to 15 minutes was ineffective; 2 per cent for 25 to 30 minutes killed all or almost all the cells; two per cent for 35 minutes destroyed them all.

(6) SODIUM BENZOATE. Up to 5 per cent (and also 5 per cent liquid soap) for 30 minutes were ineffective.

TABLE VI. The effect of traces of hydrochloric acid upon the disinfecting power of water for red halophiles as indicated by their destruction on contaminated wood surfaces.

Water	pH	Time for complete destruction
unchanged	5.2	3 to 4 hours
+ 0.001% HCl	4.3	3 to 4 hours
+ 0.01 % HCl	3.3	2 to 3 hours
+ 0.1 % HCl	2.4	2 to 3 hours
+ 1 % HCl	1.3	30 to 45 minutes

INCREASED LAG AT MAXIMUM SURVIVAL TIME

There is one interesting observation that was evident throughout all the tests recorded in this paper. In every instance, as the organisms approached their maximum survival time in the various solutions there was a marked increase in

the time required to re-establish growth when they were transferred to the surface of the salt agar medium. Where the cells survived in brine for very short periods, this increase in lag became apparent at once.

In the tests using plain brine solutions or brine plus proteins at temperatures below 37° the increase in lag approximately doubled the time required to produce visible growth. In some of the tests using plain water, especially at increased temperatures, or disinfectant solutions such as 2 per cent sodium hydroxide, the lag was very much greater. For example, when a heavy suspension of *Ps. salinaria #18* was put in tap water at 70°C. and 1 ml. withdrawn each minute, the following times were required to re-establish growth on salt agar:

Time in water at 70°	Time required for visible growth on salt agar medium at 42°
0 min.	2 days
1 min.	8 days
2 min.	14 days
3 min.	17 days
4 min.	25 days
5 min.	no growth at 90 days
6 min.	no growth at 90 days

This phenomenon of increased lag may be the result of ageing or injury to the surviving cells, or it may be the result of a greatly diminished number of cells requiring a longer incubation period to initiate growth. More probably it is the result of a combination of both.

DISCUSSION

Although it has been shown by Stuart (1938) and Hess (1942) that the range of salt tolerance for the growth of red halophiles can be changed by gradual adaptation, these organisms normally are absolute halophiles. The mechanism of the phenomenon of halophilism remains unknown. At one time it was believed to be a simple matter of osmotic pressure. Hess (1942) observed that by replacing Na+ ions with other cations growth was inhibited, and Gibbons and Robinson (1950) have pointed out that the salt content of halophilic cells is only a fraction of that of the medium in which they are grown. These latter workers suggest that some energy mechanism, probably associated with glycolysis, operates to exclude the salt from the cell. This may explain why some organisms can survive in concentrated brines, but it throws little light on why such concentrations are necessary for the growth of the absolute halophiles.

The results recorded here have shown that high concentrations of salt are necessary not only for growth, but also for survival. In the absence of nutrients the maximum survival time is in saturated brine, and may extend up to three or four months or more. At the other extreme, even a very heavy suspension in tap water will not live longer than four to six hours at 25°. Temperature, hydrogen ion concentration and added proteins all have a marked effect on the length of the survival period. Stuart (1940) has shown that the effect of protein concentration, pH and sodium chloride concentration on the growth of halophiles are dependent upon each other. Lochhead (1934) has shown that *Ps. salinaria* grew slowly or not at all in salt media having peptones and yeast extract as the chief source of nutrients. We have observed that although peptone has a protective action on halophilic cells in weak brines, their subsequent growth is much slower than with similar tests where blood serum, gelatin or certain other protein materials replaced the peptone.

The results from the tests showing the survival of the halophilic organisms in various disinfectants appear to be at variance with the observations of Hess and Gibbons (1942). They found that 1 per cent formaldehyde for one minute, 2 per cent lye for two minutes and 0.5 per cent available chlorine for one minute were effective in destroying red halophilic bacteria. We have found that 5 per cent formalin for 20 to 30 minutes was ineffective, and that 2 per cent lye required, not 2, but 30 to 35 minutes to destroy the cultures completely.

We believe that at least part of the explanation for these differences lies in the techniques that were used. In the tests described above, at the end of the period that the wood was immersed in the disinfectant solution it was immediately shaken in a liter of sterile 16 per cent brine. Any disinfectant remaining on the sticks would be diluted several thousand times. This rinse solution was then further diluted from 1 to 10^6 times before plating out on the salt agar medium. In many instances the direct transfer of 5 ml. of the rinse solution to plates resulted in no growth, whereas further dilution from the same liquid yielded growth. This indicated a bacteriostatic action from traces of the disinfectant carried over to the culture medium. This was particularly noticeable with formaldehyde and the quaternary ammonium compounds.

It should also be pointed out that there is a considerable difference in the resistance, not only between cultures of *Sarcina* and *Pseudomonas*, but also between different strains of *Pseudomonas*. For example, it required more than three times the exposure time in 2 per cent lye to destroy cultures of *Ps. salinaria* #18 as it did to destroy strain #2.

PRACTICAL APPLICATIONS. Experience has often shown that there may be a considerable difference in the results obtained in controlled laboratory experiments from those obtained by the application of similar procedures under the varying conditions as they exist in the commercial plants. At best, the small-scale controlled experiments are useful to indicate approximately what we might expect in their application to plant practice.

The results of the tests recorded in this paper emphasize the resistance of the red halophiles to some of the disinfectants commonly recommended for their destruction. They also suggest the more frequent and more abundant use of fresh water, and where possible, hot water. Not only does water remove the salt necessary for the continued growth of these halophiles, but when the salt content has been sufficiently reduced, it also becomes an active germicidal agent.

SUMMARY

(1) Red halophiles survive longest in saturated salt solutions and their survival time decreases as the salt content is reduced.

(2) Decreased temperature, increased pH and the addition of protein materials, prolong the survival time of halophiles in solutions of sodium chloride.

(3) Heavy suspensions of red halophiles on brine-soaked pieces of wood are destroyed by 16 to 20 hours' immersion in sea water or 4 to 6 hours in tap water.

(4) The value of water as a destructive agent for red halophiles increases as the temperature is raised.

(5) The resistance of red halophiles to common disinfectants is greater than has been indicated by previous tests.

(6) Plain tap water can be used advantageously as a supplement to the methods now advocated for controlling red contamination in fish plants.

REFERENCES

CASTELL, C. H. *Fish. Res. Bd. Can., Prog. Rep. Atl.,* No. 50, 9–13, 1950.
GIBBONS, N. E. *J. Biol. Bd. Can.*, **3**, 70–76, 1937.
GIBBONS, N. E., AND J. ROBINSON. *Proc. Soc. Am. Bact.*, p. 131, 1950.
HESS, E. *J. Fish. Res. Bd. Can.*, **6**, 10–16, 1942.
HESS, E., AND N. E. GIBBONS. *J. Fish. Res. Bd. Can.*, **6**, 17–23, 1942.
LOCHHEAD, A. G. *Can. J. Res.*, D, **10**, 275–286, 1934.
STUART, L. S. *Food Res.*, **3**, 417–420, 1938.
J. Agric. Res., **61**, 267–275, 1940.
STUART, L. S., AND L. H. JAMES. *J. Bact.*, **35**, 381–396, 1938.

The Water Transfer in Codfish Muscle Immersed in Sodium Chloride Solutions

By H. Fougère
Gaspé Fisheries Experimental Station,
Grande Rivière, P.Q.
(*Received for publication February 7, 1952*)

ABSTRACT

Codfish muscle will imbibe water as long as the average concentration of the brine within the tissue does not exceed approximately 10 per cent. Beyond this concentration it loses water.

Reay (1935) studied salting as applied to herring by following the change in weight of herring fillets immersed in salt solutions. He showed in his experiments that after 10 days herring muscle takes up water and therefore gains weight in salt solutions of concentrations up to 18 per cent at 0°C. Likewise, Callow (1931) showed that pork muscle takes up water from a solution containing as much as 20 per cent salt at 0°C. Tarr (1940, 1942) observed that fillets of flounder treated in weak brine increase up to 3 per cent in weight. He attributes this increase in weight to the swelling of the proteins in salt solutions. Chipman (1930) found that the weight of codfish muscle soaked in fresh water increases up to 30 per cent of its original weight. This capacity to absorb water is a property of dead tissues and recalls to mind the experiment (Thomas, 1946) describing the swelling of dead frog muscle in a hypertonic sodium chloride solution.

It is proposed in this investigation to study the behaviour of water in codfish muscle as affected by sodium chloride. This is accomplished by following the change in weight of samples of muscle salted in brines of various concentrations. An attempt is also made to explain the mechanism whereby water is set free during the salting process.

EXPERIMENTAL

Unless otherwise mentioned, all experiments throughout this investigation were carried out at approximately 20°C.

All samples were obtained from freshly caught fish.

The salt used was C.P. analytical sodium chloride.

The chlorides in the tissues were determined by the dichlorofluorescein method as outlined by Dyer (1943).

The moisture contents were determined by measuring the loss in weight due to evaporation of moisture from weighed samples heated in an oven at 105°C. for 24 hours.

All per cents as regard salt solutions are here referred to as grams of sodium chloride per 100 grams of solution.

As a general rule the salting technique consisted in immersing weighed samples in dilute brines for given periods of time. The change in weight due to

J. Fish. Res. Bd. Can., 9 (8), 1952.
Printed in Canada.

salting was recorded. The procedure is similar to that used by Chipman (1930) and although no comments can be made as to the accuracy involved in weighing the samples either before or after salting, repeat experiments have given the same relative results.

RESULTS

The samples were equal size cubes each weighing approximately 25 grams. Their moisture content was 80 per cent. One cube of known exact weight was added to each of several beakers containing 50 grams of the same salt solution of known concentration. At successive intervals, one sample was withdrawn from its beaker, set aside to drip for about two minutes then weighed.

The change in weight as observed is due apparently to the proportionality at which the salt from the solution and the water from the fish are exchanged. If the weight of the salt absorbed were deducted from the change in weight at one particular instant, the resultant would be the weight change due to water only.

TABLE I. Change in weight K, due to loss or gain of water, and corresponding concentration of brine $\frac{Z}{Y+Z}100$ within the tissue when samples of codfish muscle are salted in different weights of sodium chloride solutions.

W	P_0	P_s	C	K	Y	Z	$\frac{Z}{Y+Z}100$	$\frac{S}{G+W}100$
Weight of salt solution grams	Original weight of sample—grams	Weight of sample after salting—grams	Per cent total change in weight	Per cent change in weight due to water only	Per cent moisture in salted sample	Per cent salt in sample	Per cent conc. of brine within the sample	Per cent theoretical conc. of ambient solution
Samples salted in 10% salt solutions								
500	25.49	30.25	+ 18.6	+ 9.9	78.8	7.43	8.6	9.6
1000	25.30	29.98	+ 18.3	+ 9.1	78.5	7.90	9.0	9.1
2000	25.48	30.13	+ 18.3	+ 8.9	78.6	7.87	9.9	9.8
Samples salted in 12% salt solutions								
50	24.65	28.85	+ 17.0	+ 9.0	79.25	6.9	8.0	8.5
75	25.81	30.07	+ 16.5	+ 7.0	78.60	7.8	9.2	9.3
200	26.38	25.41	− 3.3	− 11.9	76.3	8.6	10.1	10.8
600	24.27	23,42	− 3.5	− 12.1	75.5	9.4	11.0	11.6
Samples salted in 14% salt solutions								
100	25.70	27.62	+ 7.4	− 1.9	75.70	8.7	10.3	11.6
200	24.81	22.44	− 9.3	− 14.7	71.8	10.2	12.4	12.7
100	98.31	122.18	+ 24.3	+ 15.9	79.4	7.0	8.1	7.9
100	88.89	113.21	+ 27.3	+ 18.8	79.0	6.7	7.8	8.1
100	96.39	114.54	+ 18.8	+ 10.4	79.5	7.1	8.0	7.9

Therefore the results were expressed according to the following formula which represents the change in weight due to the gain or loss of water:

$$K = \frac{P_s - P_0 - H}{P_0} 100$$

where K = per cent change in weight due to water only
P_s = weight of sample after salting
P_0 = initial weight of sample
H = grams of salt in sample

When K is negative the sample has lost water, when positive it has imbibed some. Figure 1 illustrates the change in weight due to the gain or loss of water only. A change in weight in this case represents a change in the direction of the transfer of water either from the ambient solution into the muscle or vice versa.

Since the internal salt concentration of a sample varies with the weight of the salt solution, experiments were conducted by salting samples of approximately 25 g. in different weights of different solutions ranging in concentration from 10 to 14 per cent. Furthermore, larger samples approaching 100 g. in weight also were salted in 100 g. of a 14 per cent salt solution. The salting period was approximately 20 hours. The results are shown in Table I.

DISCUSSION

According to Callow (1930) and Osborne (1914) protein systems such as gelatin gels and egg yolks exhibit phenomena similar to those observed with cod-fish muscle. An explanation for the absorption of water is sought in the behaviour of proteins in salt solutions. As salt diffuses through the fish tissue, a protein salt and water system is set up in which the proteins swell to a degree corresponding to the quantity of salt present. Since the imbibition capacity of the proteins increases with swelling, not only will they retain the water already present in the system but may imbibe some from the external solution.

Moreover, it is universally acknowledged that at equilibrium, the salt-water ratio in the fish muscle is the same as that of the ambient salt solution. Therefore if at that stage, the internal salt concentration (concentration of the brine within the tissue) is $\frac{Z}{Y+Z}100$, and the external or ambient concentration is $\frac{S}{G+W}100$, then $\frac{Z}{Y+Z}100 = \frac{S}{G+W}100$

Z = the salt content of the fish sample
Y = the moisture content of the salted sample
S = weight of the salt in the solution used
G = weight of the available moisture in the original sample
W = weight of the solution used

Hence it is possible to calculate the maximum attainable internal salt concentration of a given sample salted under given conditions. It is furthermore possible to calculate the internal and external salt concentrations at any time during the

process of salt and water diffusion as each side of the equation holds true at any instant during the salting process, although they are equal only at equilibrium.

The internal salt concentration of the samples in the 14 per cent salt solutions (Figure 1) therefore cannot exceed 10 per cent, yet water is given up. Likewise, the maximum final concentration attainable in the 12 per cent salt solutions is 6.6 per cent and water has been imbibed. On this basis a 100 g. sample immersed in 100 g. of a 14 per cent salt solution should imbibe water since the internal salt

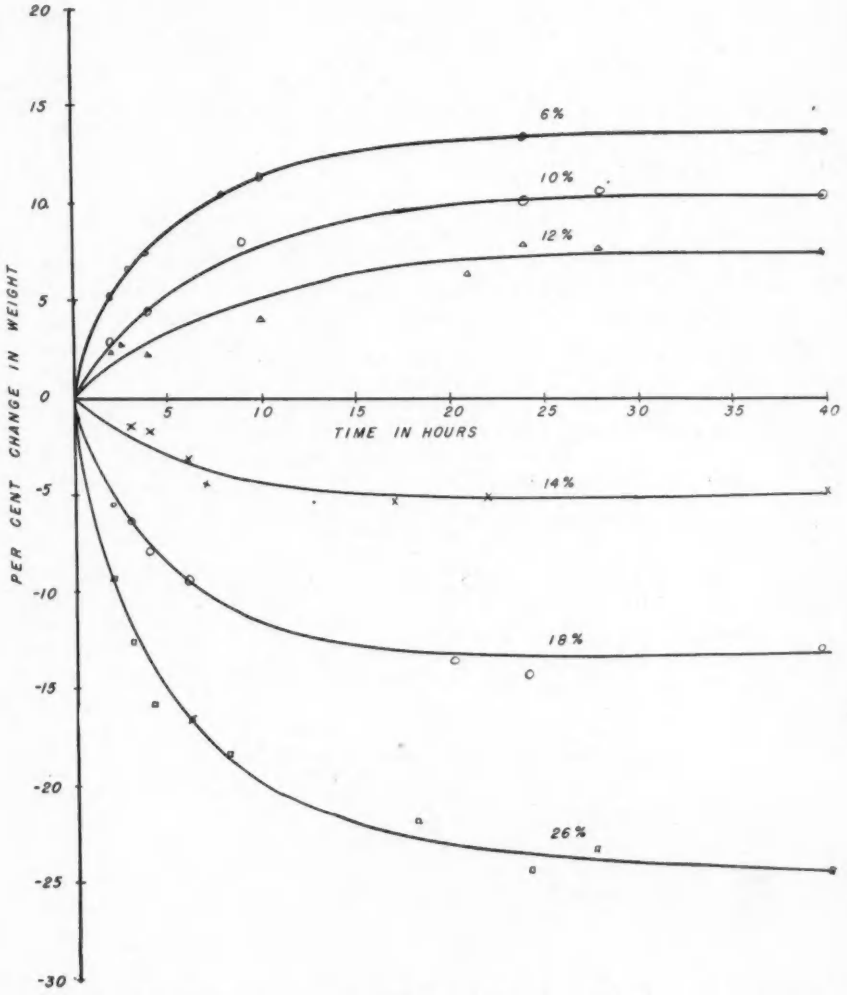


FIGURE 1. Change in weight of codfish muscle immersed in brines of the concentrations indicated on each curve.

concentration under such conditions cannot be greater than 7.7 per cent, and the data shown in Table I seem to verify this point.

In all cases as may be seen in Table I, K (the weight change due to water) is positive when $\frac{Z}{Y+Z}100$ (the internal salt concentration) is less than 10 per cent. It is negative when $\frac{Z}{Y+Z}100$ is 10 per cent or greater. That is to say, as long as the internal salt concentration has not reached a value near 10 per cent the fish absorbs water. When it reaches 10 per cent or more it gives up water. This conclusion seems to hold true irrespective of the weight of the sample and that of the salt solution.

These results correspond closely to those of Dyer (unpublished results) that myosin, which constitutes approximately 75 per cent of the total muscle proteins of codfish, undergoes denaturation in an 8 to 10 per cent sodium chloride solution.

SUMMARY AND CONCLUSION

Experiments tend to show that codfish muscle immersed in salt solutions will either absorb or give up water according to the quantity of salt which diffuses within. It will imbibe water provided the average concentration of the brine within the tissue does not exceed approximately 10 per cent. Beyond this concentration the fish muscle loses water.

These results, coupled with information available from other sources, lead to the assumption that the direct effect of salt on the removal of water from fish in salt solutions is to release water held as water of imbibition by the proteins. This is accomplished when sufficient salt has diffused within the shell of water surrounding the protein micelles to establish a concentration of approximately 10 per cent or more.

In salt solutions of 10 to 12 per cent concentration, surface proteins must be denatured and thus give up water; but others at some depth within the tissue, by reason of a concentration gradient, must imbibe a weight of water which exceeds that lost by the denatured ones.

REFERENCES

Callow, E. H. *Report U. K. Food Investigation Bd.*, 74–76, 1930.
Report U. K. Food Investigation Bd., 144–147, 1931.
Chipman, H. R. *Ms. Rep., Biol. Bd. Can.*, No. 32, 1930.
Dyer, W. J. *Ind. Eng. Chem. Analytical Ed.*, **15**, 439, 1943.
Osborne, W. A. and H. E. Kincaid. *Biochem. J.*, **8**, 28, 1914.
Reay, G. A. *Report U.K. Food Investigation Bd.*, 70–75, 1935.
Tarr, H. L. A. *J. Fish. Res. Bd. Can.*, **5**, 411–427, 1942.
Fish. Res. Bd. Can., Prog. Rep. Pac., No. 43, 10–13, 1940.
Thomas, P. *Manuel de Biochimie*, 2e édition, 999 pp. Paris: Masson et Cie, Editeurs, 1946.

A Study of the "Lipoxidase" in the Flesh of British Columbia Herring[1]

By M. M. R. Khan[2]

Department of Zoology, University of British Columbia

(*Received for publication December 10, 1951*)

ABSTRACT

From the dark muscle of British Columbia herring a highly active enzyme capable of peroxidizing non-conjugated unsaturated fatty acids was isolated. This "lipoxidase", which was shown to be a nitrogenous complex possessing no heavy metals or sulphydryl group as the active centre, is heat-labile and can act only in presence of activators such as certain iron-containing organic nitrogenous compounds. Two such compounds, namely haemoglobin and cytochrome "c" were isolated. The enzyme exhibits optimal activity at 15°C. and pH 6.9. There is also an optimal concentration of enzyme, substrate, and of the activators for maximal enzyme activity. The presence of the activators appears to change the kinetics of the reactions. The inhibition of the enzymic reaction brought about by cyanide and azide is possibly due to the inactivation of the iron-containing activators rather than of the enzyme itself.

INTRODUCTION

The deterioration in quality resulting from rancidity that sometimes develops in fatty foods during storage causes considerable loss to all those concerned in general with the production, processing, storage, sale or consumption of such products. The problem is particularly important to the fisheries industries because of the high fat content of many fishes and more particularly because of the compositions of fish fats. These fats are especially rich in unsaturated fatty acids which are markedly prone to oxidative rancidity during storage.

In frozen fish the oxidation of the indigenous fats is accompanied by such undesirable changes as a yellow or brown discoloration of exposed fats (rusting), bleaching of the natural red astacin pigments (in salmon), and production of unpleasant flavours variously described as rancid, bitter, "salt-fish", and by similar descriptive terms. The off-flavours are known to be caused by the compounds formed as a result of oxidative break-down of fats. Various products have been identified in autoxidized lipides. Among the more common are peroxides, peracids, ketones, aldehydes, substituted ethylene oxides, carboxyl compounds, hydroxyl compounds, water, and carbon dioxide (Black, 1945; Mattil, 1941). In general, however, these studies have been concerned with fats that were oxidized far beyond the stage where rancidity first appeared.

Conditions affecting the onset of rancidity in fats have occupied the attention of many investigators during the past few years. The development of ran-

[1]Adapted from a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology, University of British Columbia. Prepared for publication by Dr. Neal M. Carter, Pacific Fisheries Experimental Station, Fisheries Research Board of Canada.

[2]Present address: Central Fisheries Department, Karachi 3, Pakistan.

J. Fish. Res. Bd. Can., 9 (8), 1952.
Printed in Canada.

cidity in fatty foods may be attributed to both external and internal factors. Among the former are micro-organisms, temperature, moisture, atmospheric oxygen, and light; while among the latter are proteins, enzymes, co-enzymes, minerals of the tissue, and chemicals which may be added during processing as well as materials (e.g. metal foils) composing the package. The effects and control of micro-organisms have been reviewed in detail (Lea, 1939) and the effects of curing, different curing mixtures, and impurities in the curing salts have been extensively studied (Lea, 1931, 1933, 1939; Hills and Conochie, 1945; Hills, 1946; Greenwood, Striter and Kraybill, 1945; Oser, 1946).

A factor comparatively little investigated but probably of the first order of importance is the effect of the enzymes or enzyme systems in fatty tissue. Banks (1937, 1944) demonstrated the presence of a heat-labile system in herring muscle which stimulated rancidity in extracted herring oil. Lea (1931, 1933) suggested that an enzyme system might be responsible for the rapid deterioration of bacon and later (1937) presented evidence for its presence. Watts and Peng (1947) have compared the catalytic effects of pork muscle extracts and haemoglobin on peroxidation of lard. Other enzymes capable of oxidizing fatty acids and fats have been reported. Munoz and Leloir (1943) prepared from liver an enzyme that catalyses the oxidation of low-molecular-weight fatty acids, work which has been considerably extended by Lehninger (1945) and Lehninger and Kennedy (1948). Lang's (1939) results are also of interest in this connection. Hove (1943) found that rat liver and gastric mucosa contained an oxidase capable of oxidizing unsaturated fats, but the intestine and muscle were inactive. Extract of gastric mucosa retained its activity at pH 3. The capacity of this extract to catalyse reactions at such a high hydrogen ion concentration is possibly due to the fact that the enzyme involved is produced in an environment, namely the gastric mucosa, characterized by high acid production. Süllmann (1947) has demonstrated in the striated muscle of the rabbit the presence of an enzyme that strongly peroxidizes unsaturated fatty acids and fats. Brocklesby and Rogers (1941) reported that an extract of salmon liver commenced destruction of vitamin A in dogfish liver oil within 45 minutes at room temperature and had destroyed 20 per cent of the vitamin at the end of 36 hours at that temperature, an effect they attributed to the direct or secondary action of a lipoxidase. Reiser (1949) gave evidence of the presence of two heat-labile factors in bacon which may be responsible for the development of rancidity in bacon.

A "lipoxidase" present in legumes was investigated and used in a study of the mechanism of the oxidation of isolated unsaturated fatty acids (Bergström, 1945; Holman, 1947). The literature on this enzyme has recently been reviewed (Holman, 1948).

However, the effects of the tissue enzymes in fatty foods on the development of rancidity have not been adequately studied. The investigations of Lea (1937), Watts and Peng (1947), and Banks (1944) were not carried to a stage where the nature of the enzyme or enzyme systems could be conclusively ascertained. A recent investigation by the author, dealing with antioxidant glazes for frozen fish, resulted in some interesting findings that led to the present more detailed

study of the enzyme system involved in the onset of rancidity in the flesh of an oily fish. During the course of this work a report of Banks' investigations in 1939 concerning the influence of haematin compounds on the oxidation of an unsaturated fatty acid substrate (linoleic acid), published in 1949, became available. This is referred to later on page 413.

MATERIAL AND METHODS

MATERIAL. Herring, *Clupea pallasi*, were chosen as material because of their relatively rich fat content and the nature and extent of the unsaturation of the fats. High unsaturation of herring oil has been known to provide suitable conditions for rancidity to progress with a fair degree of rapidity. As a result the proneness of herring flesh to undergo oxidative rancidity is most likely due to the activity of the lipoxidase in the flesh. Available information regarding lipoxidase activity in fish flesh records the presence of such an enzyme only in herring flesh. Banks (1937), studying the enzyme system influencing rancidity in herring, reported that the enzyme is mostly concentrated in the dark streak of muscle along the lateral lines of the fish and that the major portion of the flesh exhibits either very little or no lipoxidase activity at all. These findings were substantiated during some preliminary tests in the present investigation. Samples of the dark strip of muscle from British Columbia herring exhibited some 7.5 times more lipoxidase activity than did like portions of the remaining flesh. Hence the dark streak of herring flesh was used for extracting the enzyme.

SUBSTRATE. In order to find a workable method for assaying the lipoxidase activity of herring muscle it was necessary at first to ascertain the essential substrate for the enzyme.

Sumner and Sumner (1940) observed that the lipoxidase in legumes oxidized carotene only in presence of unsaturated fats. This observation was later confirmed by Tauber (1940). Sumner (1942b) submitted evidence that carotene is not oxidized either directly or indirectly by the peroxides formed from unsaturated fats. It was also shown that the oxidation of carotene requires that the peroxidation of unsaturated fat be actually in progress. Sumner (1942a), investigating the effect of lipoxidase on various unsaturated compounds, reported that the enzyme is most effective on the unsaturated part of a typical carbon chain structure

$$-CH_2-CH=CH-CH_2-CH=CH-(CH_2)_7-COOH$$

where the configuration of the groups about the double bond nearest to the carboxyl group is probably of the *cis* type. Similar results were reported by Holman and Burr (1945) and Holman and Elmer (1947). These authors, using purified fatty acids and their esters, found (1) that lipoxidase attacked linoleic acid, linolenic acid, their esters, and methyl arachidonate; (2) that linoleate, linolenate, and arachidonate esters were oxidized at the same rate; (3) that only the *cis* isomers of linoleic and linolenic acids were attacked. From these results it is suggested that the essential substrate structure for attack by legume lipoxidase seems to be the methylene-interrupted, doubly unsaturated system

$$-CH_2-CH=CH-CH_2-CH=CH-CH_2-$$

with a *cis* configuration about both double bonds. A repetition of the above studies with herring lipoxidase, using oleic, linoleic, and linolenic acids as substrates, produced results which indicate that the essential substrate structure for herring lipoxidase is similar to that for legume lipoxidase.

Oleic acid (*cis*) was peroxidized by herring flesh extract very slowly and the maximum effect, obtained in 20 minutes, was less than 20 per cent of the theoretical. Ordinary linoleic acid (both double bonds with *cis* configuration) was peroxidized with great rapidity, the maximum being over 80 per cent of the theoretical total peroxidation of one double bond. Linoleic acid with one double bond having a *trans* configuration (obtained from Hormel Foundation, Austin, Minn., U.S.A.) was peroxidized at a rate about a third of that observed in case of ordinary linoleic acid, the final amount of peroxide formed also being about one third that from the *cis-cis*-linoleic acid. Linolenic acid (*cis*) was peroxidized at a rate appreciably higher than that for ordinary linoleic acid and the maximum was about 110 per cent of the theoretical total peroxidation of one double bond.

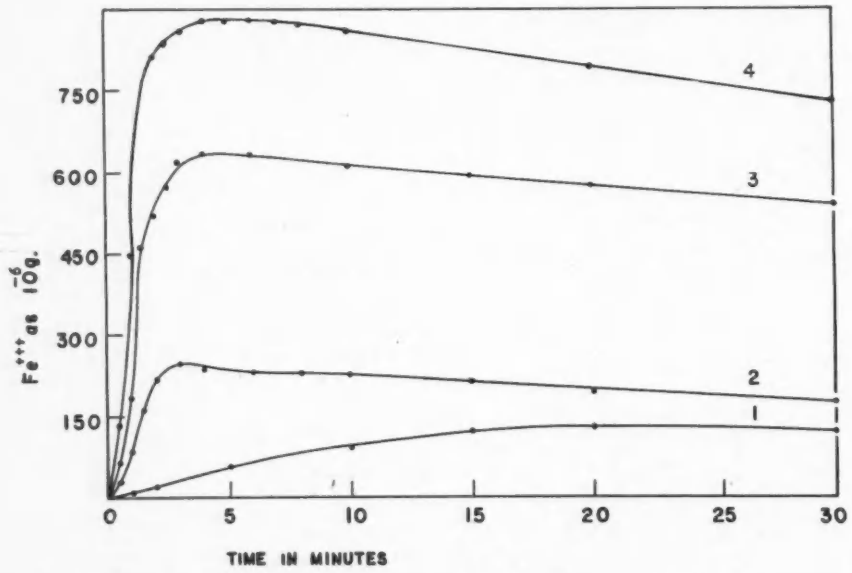


FIGURE 1. Activity of 0.01 mg. crude lipoxidase enzyme from brown muscle of herring, assayed by ferric thiocyanate method against 2 mg. of substrate (1) oleic acid, (2) *cis-trans*-linoleic acid, (3) *cis-cis*-linoleic acid, (4) *cis*-linolenic acid.

Figure 1 illustrates the rates and amounts of activity displayed by 0.01 mg. of crude herring lipoxidase acting on 2 mg. each of the foregoing unsaturated fatty acid substrates. Activity was measured by the ferric thiocyanate method mentioned below under assay methods.

From these experiments it was decided that the readily available ordinary (*cis-cis*) linoleic acid was a suitable substrate for use in following the activities during the particularized examination of the nature of the lipoxidase.

ASSAY METHODS. Various methods of assay of lipoxidase, involving its different properties, have been suggested. These methods can be divided into the following classes: (1) those which employ the Warburg apparatus and measure the absorption of oxygen during the oxidation of the unsaturated substrates (Craig, 1936; Banks, 1949; Hummel and Mattil, 1944; Süllmann, 1941; Tauber, 1940); (2) methods in which the peroxides formed are estimated (Balls, Axelrod and Kies, 1943; Kokatnur and Jelling, 1941; Sumner and Dounce, 1939; Sumner and Somers, 1944; Sumner, 1943; Süllmann, 1941; Young, Vogt and Nieuwland, 1936); (3) methods based on decolorization of a suspension of carotenoid compounds containing unsaturated fat (Balls, Axelrod and Kies, 1943; Reiser and Fraps, 1943; Sumner and Sumner, 1940; Sumner, 1942a,b). Sumner (1943) employed a ferric thiocyanate method for determination of the peroxide products. More recently Sumner and Smith (1947) used an assay based on the destruction of bixin, a carotenoid dicarboxylic acid.

In comparatively old methods of measuring peroxide products, the substrates used were often not homogeneous. The recent methods employing linoleic acid or its esters as substrate ensure homogeneity, also a favourable degree of dispersion of the substrate. The latter is important since the rate of oxidation of the unsaturated compounds is influenced by the degree of dispersion of the substrate.

At the commencement of the present investigation the method of Sumner (1943), using linoleic acid suspension as substrate and the ferric thiocyanate method for determination of the peroxide products, was employed for estimating lipoxidase activity of the herring flesh. Although results with this method were satisfactory for some of the preliminary work, it later had to be discarded because of its disadvantage that a side reaction rather than the primary phenomenon of hydroperoxide formation from the unsaturated fatty acids is being measured. The bixin decolorization method of Sumner and Smith (1947) was tried, but herring flesh extract failed to decolorize bixin at pH 4–9.

In the course of investigations of the spectra of autoxidized fatty acids, Holman and Burr (1945) studied the spectra of lipoxidase-oxidised fatty acids. They noted that linoleic acid, ethyl linoleate, linolenic acid, ethyl linolenate, and methyl arachidonate showed increased absorption near 2300 and 2700 Å, indicating that these substances were changed by lipoxidase. The products which gave the increased absorption were considered to be the conjugated unsaturated carbonyl compounds. A method of assay was developed by Theorell, Bergstrom and Åkeson (1946), making use of the absorption of the primary products of oxidation of unsaturated fatty acids. This method overcomes the disadvantages of the previously mentioned ones and has the added advantage of being a very quick and reproducible method. A homogeneous substrate solution is used, and the products of the reaction are measured spectrophotometrically. Under these conditions, peroxide production is proportional to time and to enzyme concentration over wide ranges.

This spectrophotometric method, with certain modifications, was adopted in the present work. The wavelength of light for maximal absorption by the primary products of oxidation of linoleic acid in presence of herring lipoxidase was found to be 2380 Å (Figure 2) instead of 2340 Å as employed by Theorell *et al.* (1946) in standardization of soybean lipoxidase. The temperature (15°C.) and pH (6.9) employed were those optimal for herring lipoxidase. Furthermore, a larger quantity of ethyl alcohol (5.0 ml.) was necessary to stop the enzyme reaction as against 2.0 ml. used by Theorell *et al.* The details of the modified method are as follows.

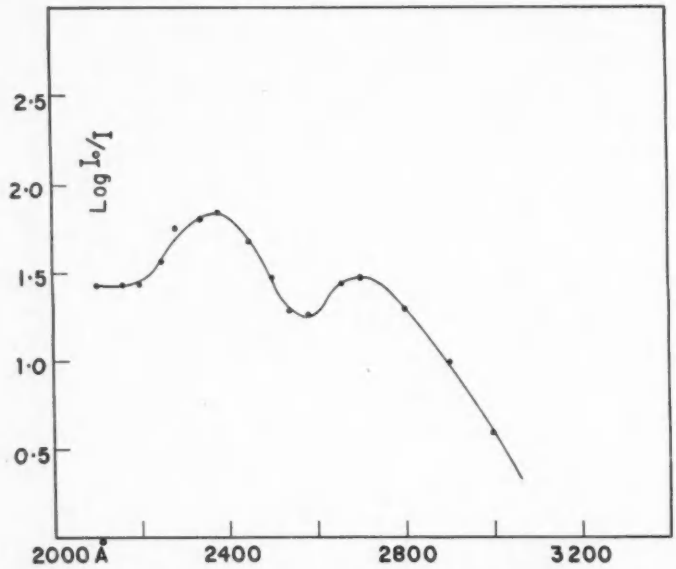


FIGURE 2. Absorption spectrum of the primary oxidation products of linoleic acid in herring lipoxidase catalysed reaction. (Two mg. of linoleic acid and 0.01 mg. of active enzyme solution.)

One millilitre of 0.200 per cent *cis-cis*-linoleic acid in 0.2 M acetate buffer of pH 6.9 is placed in a Warburg respiration bottle; into the side arm is put 0.2 ml. of enzyme solution. The bottle is filled with oxygen and placed in a water bath at 15°C. After temperature equilibrium is reached, the contents of the bottle are mixed and the mixture shaken well. At the end of two minutes 5.0 ml. of 95 per cent ethyl alcohol (free from ammonia and oxides of nitrogen) is added to stop the reaction. The mixture (6.2 ml.) is diluted with 75 per cent ethyl alcohol of the same purity to a strength suitable for measurement of its absorption at 2380 Å with a Beckman spectrophotometer. The blank is prepared in the same manner except that the enzyme is inactivated by boiling prior to its incorporation into the reaction mixture.

A unit of lipoxidase will be defined as that amount of the enzyme which under the above experimental conditions produces in the final mixture (6.2 ml.) an optical density of 2 at the end of two minutes of reaction.

EXTRACTION AND PURIFICATION OF ENZYME. The conditions for the extraction and purification of the enzyme were ascertained by a series of exploratory experiments carried out with a lot of Point Grey herring caught in May, 1948, off Burrard Inlet close to Vancouver, B.C. Results recorded here were obtained with a later lot of herring caught in August, 1948, along the coast of British Columbia, about 100 miles north of Vancouver. Both lots were frozen and stored before extraction was performed. It was observed that there was a noticeable difference between the lipoxidase activity of the two lots of herring (Table I). This difference might have been caused by variable factors such as locality of catch

TABLE I. Lipoxidase activity of the dark muscle of two different lots of herring. (NOTE: Each sample represents ten herring taken at random from the lot concerned. Storage conditions for Lots Nos. 1 and 2 were similar within narrow limits of temperature and elapsed storage time.)

Lot	Sample No.	Amount of Fe^{+++} as 10^{-6} gm. produced per 100 gm. tissue (ferric thiocyanate assay)	
Lot No. 1 (1000 herring caught in May, 1948)	1	146	av. = 140.8
	2	131	
	3	157	
	4	127	
	5	143	
Lot No. 2 (2500 herring caught in August, 1948)	1	231	av. = 245.6
	2	249	
	3	261	
	4	245	
	5	242	

(e.g. water temperature in relation to metabolic activity of the fish), season of catch (e.g. maturity in relation to spawning), type of food consumed, or age. A future extension of this study might lead to interesting observations on possible relations of lipoxidase activity to certain of these factors when taken into account more completely than the present work allowed.

Fifteen kilograms of the excised dark streak of muscle were finely ground, lyophilized and extracted in the cold with low-boiling "Skellysolve" petroleum spirits. The defatted tissue was extracted in a Waring Blendor at 0°C. with acetate buffer of pH 5.6. The supernatant slightly yellowish fluid obtained by centrifuging the mixture was treated with ammonium sulphate. At 35 per cent concentration of ammonium sulphate an inactive fraction precipitated out. The concentration of ammonium sulphate in the clear filtrate was now raised to 60 per cent, whereupon an active fraction precipitated out. The precipitate was filtered

and washed with 60 per cent ammonium sulphate, dissolved in a minimum amount of water, and dialysed overnight in running water until free from sulphate. The dialysed solution was then adjusted to pH 6.8 with dilute ammonium hydroxide. The inactive precipitate was filtered off. The filtrate was neutralized with 0.1 N NaOH to pH 7, and treated with ethyl alcohol in the cold (0°C.). At an ethyl alcohol concentration between 16 and 18 per cent an inactive precipitate was obtained. The filtrate from this was dialysed and dried in vacuum and the residue dissolved in a minimum amount of water. This solution was now treated with 20 per cent basic lead acetate. With 3 volumes of lead acetate to 2 volumes of the solution, a precipitate was obtained. At this stage an interesting point was noted. The precipitate with basic lead acetate did not exhibit any activity. At the same time the activity of the soluble fraction was lowered to less than 30 per cent of its expected value. That this lowered activity was not due to oxidation or denaturation of the enzyme, or to its distribution between the precipitate and the soluble fraction, was evident from the following facts:

(1) The precipitate did not show any activity.

(2) Treatment of the soluble fraction with mild reducing agents (Zn dust and acetic acid; sodium hyposulphite $Na_2S_2O_4$) did not increase the activity of the soluble fraction.

(3) Addition of the fraction precipitated with basic lead acetate to the soluble fraction raised the enzyme activity of the latter to its expected level. That the enzyme proper was entirely in the soluble fraction was further evidenced by the fact that boiling the soluble fraction for 5 minutes completely destroyed its enzyme activity, whereas boiling had no effect whatsoever on the activating capacity of the precipitate. Henceforth the soluble fraction will be referred to as the *enzyme proper* and the fraction precipitable with basic lead acetate as the *activator*.

In later experiments the non-dialysable activator was broken into dialysable and non-dialysable fractions. However, the activity was entirely in the dialysable fraction. The dialysable portion gave negative biuret, Millon's, Molisch and ninhydrin reactions, but positive tests for organic nitrogen. Tests for sulphur, halogens and phosphorus were negative. The material contained less than 0.45 per cent iron. Tests for copper were negative. These results indicate that the dialysable fraction is a non-protein, iron-containing nitrogenous compound. The absorption spectra of the dialysate showed two maxima, one near 4480 and one at 5610 Å (Figure 3).

Experiments were repeated to obtain the activator without the use of lead acetate, since attempts to dissolve the activator from the lead acetate precipitate resulted in a breakdown of the activator into a dialysable non-protein and a non-dialysable protein component. The dispensing of the use of lead acetate was effected according to the following scheme.

After separation of the precipitate obtained by the use of 16 per cent ethyl alcohol, the concentration of alcohol was raised to 60 per cent (at 0°C.). A reddish yellow precipitate was obtained. This showed all the properties of the activator.

Accordingly in the final purification the fractionation with basic lead acetate

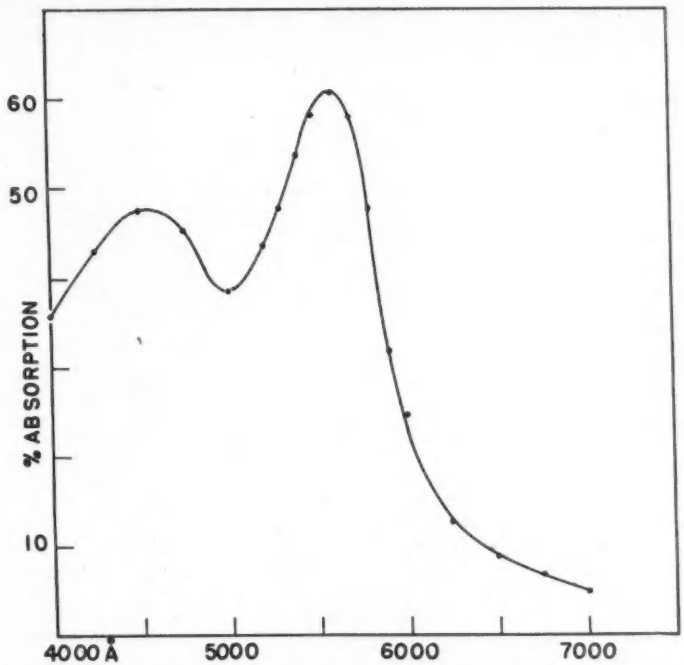


FIGURE 3. Absorption spectrum of the dialysable breakdown product of activators. (Concentration of substrate, 0.2%.)

was discarded and the activator fraction was separated from the enzyme by precipitation at 60 per cent concentration of ethyl alcohol.

ENZYME PROPER. After separation of the activator fraction, the alcohol was removed by vacuum distillation at room temperature. The pH of the aqueous solution was adjusted to 6.8 with 0.2 M acetate buffer; sodium chloride was added

TABLE II. Lipoxidase activity at different stages of fractionation of the extract.

Fractionation	Total mg. N_2	Total units	Units/mg. N_2	
1. 60% Ammonium sulphate	40,000	2,040,000	51	
2. pH 6.8	13,160	1,250,000	95	
3. 16% Ethyl alcohol	4,835	870,000	180	
4. Precipitation with basic lead acetate	2,250	540,000	56	*
			63	240
			121	
5. Precipitation with 60% ethyl alcohol	1,160	370,000	320	
6. 50% Sodium chloride	734	290,000	395	
7. Trichloroacetic acid	512	230,000	450	
8. Electrophoresis	80	96,000	1200	

*Separation of activator from enzyme took place at this stage.

to 50 per cent concentration, the inactive precipitate was removed, and the filtrate dialysed and lyophilized. This lyophilization was necessary from three considerations: (1) to minimize bulk, (2) to get an idea of the amount of material and (3) to preserve the enzyme until needed. The lyophilized tissue was dissolved in the minimum amount of water and 1 volume of 50 per cent trichloroacetic was added to 5 volumes of the solution. The inactive precipitate was removed, the solution lyophilized, then redissolved and subjected to electrophoresis. Progressive concentration of the enzyme at various stages of fractionation is shown in Table II.

ELECTROPHORESIS OF ENZYME. A simple apparatus was designed (Figure 4) for the purpose of electrophoresis. This proved quite satisfactory for the electrophoresis of relatively large volumes of materials, especially when the components were coloured.

FIGURE 4. Electrophoretic apparatus designed in the laboratory.

In a phosphate buffer (0.008 M NaH_2PO_4–0.064 M Na_2HPO_4) of pH 7.7, the active fraction migrated to the cathode when the applied potential was 130 volts and the current flowing through the solution was 10 milliamperes. The active fraction was pipetted out, dialysed, and dried under vacuum. This was later subjected to electrophoresis in a Tiselius electrophoretic apparatus. The results of these experiments are shown in Table III and Figure 5. The three peaks were

TABLE III. Electrophoretic analysis of herring lipoxidase.

Buffer	pH at 25°C.	Concentration of material (%)	Time of electrophoresis (min.)	Number of peaks
0.008 M NaH_2PO_4—0.064 M Na_2HPO_4	7.7	1.0	90	3
0.008 M NaH_2PO_4—0.064 M Na_2HPO_4	7.7	0.2	90	1
0.1 N NaBar—0.02 N HBar*	8.6	0.2	90	1
0.1 N NaCac—0.02 N HCac**	6.8	0.2	90	1

*Bar = diethylbarbiturate; **Cac = cacodylate.

given by the enzyme concentrate obtained after fractionation with trichloroacetic acid. Three other patterns showing only one peak each were obtained with the active fraction separated by electrophoresis in the apparatus designed in the laboratory. From the electrophoretic patterns it appears that the isolated enzyme is a single substance.

Attempts were made to crystallize the enzyme from the electrophoretically separated highly active fraction. The enzyme failed to crystallize from alcohol and acetone at 0°C. Similar attempts to crystallize the enzyme by dialysing its concentrated solution against saturated ammonium sulphate solution also failed.

PURIFICATION OF ACTIVATOR. The activator precipitate obtained at 60 per cent concentration of alcohol was dissolved in a minimal amount of water, an equal volume of 50 per cent ammonium sulphate was added, and the mixture held at 60°C. for one-half hour; a precipitate formed. The mixture was refrigerated overnight and centrifuged. Both the precipitate (*a*) and the filtrate (*b*) exhibited activating properties. The precipitate (*a*) was dissolved in water, brought to pH 6.8–7.0 with dilute ammonia, and sodium chloride added up to a concentra-

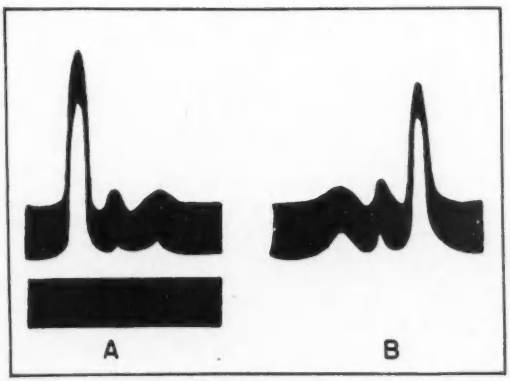


FIGURE 5. Electrophoretic pattern of enzyme concentrate obtained after fractionation with trichloroacetic acid. (A) ascending limb, (B) descending limb. The dark bar at the base of (A) represents the relative length of one arm of the cell.

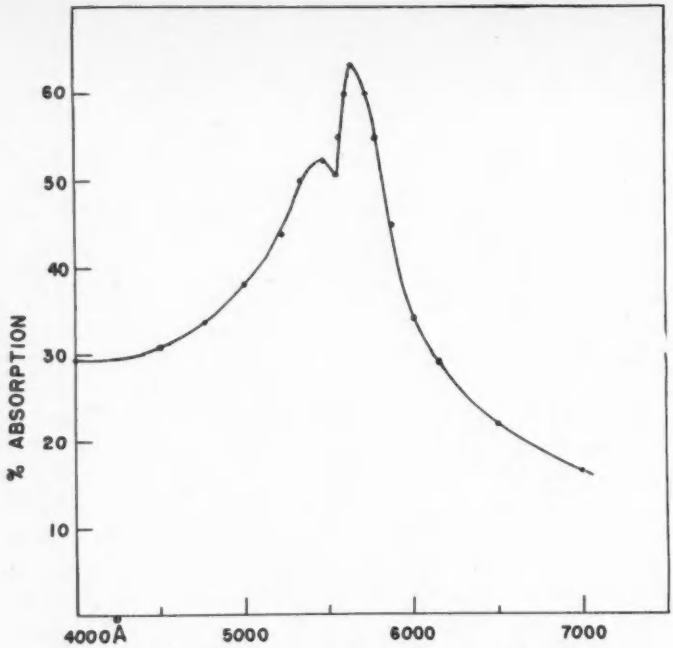


FIGURE 6. Absorption spectrum of activator (*a*). (0.2% solution.)

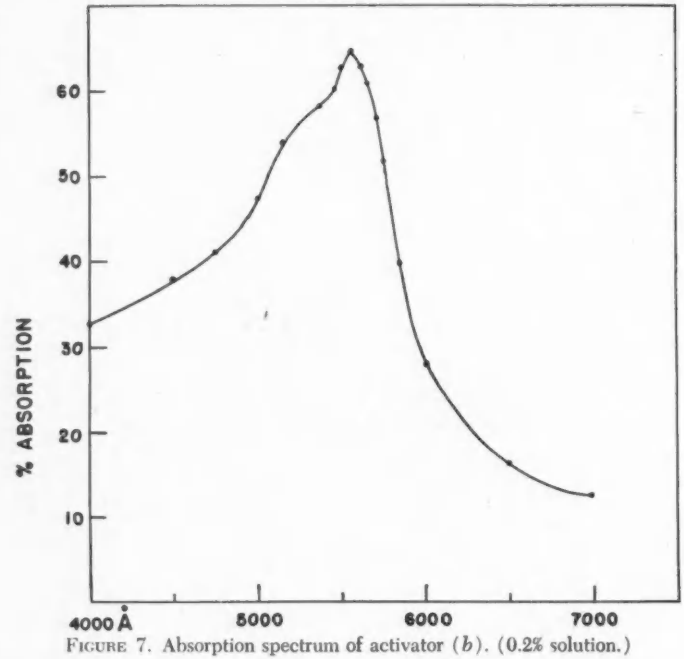


FIGURE 7. Absorption spectrum of activator (*b*). (0.2% solution.)

tion of 50 per cent. The activator precipitated out. This was centrifuged, dissolved in water and then dialysed. Biuret, Millon's, xanthoproteic, ninhydrin, and thiocyanate tests were positive. Tests for sulphur, halogens, phosphorus, and copper were negative. The material contained less than 0.30 per cent iron. This compound showed absorption maxima at 5450 Å and at 5630 Å, following reduction with $Na_2S_2O_4$. The filtrate (*b*) was treated with one-tenth of its volume of 90 per cent trichloroacetic acid and the solution was refrigerated for 2 hours, then centrifuged. The active sediment was brought into solution by stirring with 0.1 N NaOH and the solution diluted to a suitable volume with distilled water. This solution showed positive Biuret, Millon's, xanthoproteic, ninhydrin, and thiocyanate tests. Sulphur, halogens, phosphorus, and copper were absent. The iron content was less than 0.35 per cent. The absorption maximum was near 5560 Å following reduction with $Na_2S_2O_4$. The results of absorption experiments appear in Figures 6 and 7.

RESULTS

The slight activity exhibited by the enzyme fraction after removal of the activators might have been due possibly to incomplete removal of the activators, since with further fractionation it was evidenced that the enzyme alone failed to peroxidize linoleic acid, while addition of a small amount of the activator fraction restored the activity of the enzyme. It was evident, therefore, that the lipoxidase concerned acts only in conjunction with an activator or activators. The character of the activators is discussed on page 409.

As already stated, this enzyme gave positive biuret, Millon's xanthoproteic, and ninhydrin tests whereas tests for sulphur, halogens, phosphorus, iron, and copper were negative. The protein nature of the enzyme is further evidenced by its inactivation with pepsin and trypsin. The enzyme is also inactivated by boiling. As such, the lipoxidase in the dark muscle of herring flesh is a heat-labile enzyme exhibiting protein characters, which requires an activator for its activity. Table IV indicates the activity of several compounds that can act as activator although their efficiency varies from compound to compound.

TABLE IV. Relative activity of various activators in presence of 0.01 mg. enzyme and 2 mg. linoleic acid.

Activator	Optimum amount (gm.)
Dialysable breakdown product of activators (from herring flesh)	21×10^{-4}
Activator (*a*) (from herring flesh)	8×10^{-4}
Activator (*b*) (from herring flesh)	5×10^{-4}
Haematin (mammalian)	23×10^{-4}
Haemoglobin (mammalian)	10.3×10^{-4}
Cytochrome "c" (mammalian)	6.7×10^{-4}
Fe^{++}	inactive
Fe^{+++}	"

In studying the following characteristics of the enzyme system, an optimal amount of one or both activators (*a*) and (*b*) was added to the enzyme.

Effect of pH upon the Enzyme Activity

Sodium linoleate was used as substrate and the pH was adjusted to different values from 4 to 9 by the addition of NoOH or HCl. The relative activities were measured by the spectrophotometric method, the result being expressed as the optical density at 2380 Å ($\log I_0/I$) developed after a reaction time of 2 minutes. The relative activities of the enzyme at various pH values are shown in Figure 8.

It will be observed that the enzyme is most active around pH 6.9 but the activity falls much more quickly on the acid side of the pH than on the alkaline side. It is likely that this effect is due to the relative inaccessibility of substrate in the emulsion systems existing on the acid side of the pH, while high alkalinity possibly inactivates the enzyme.

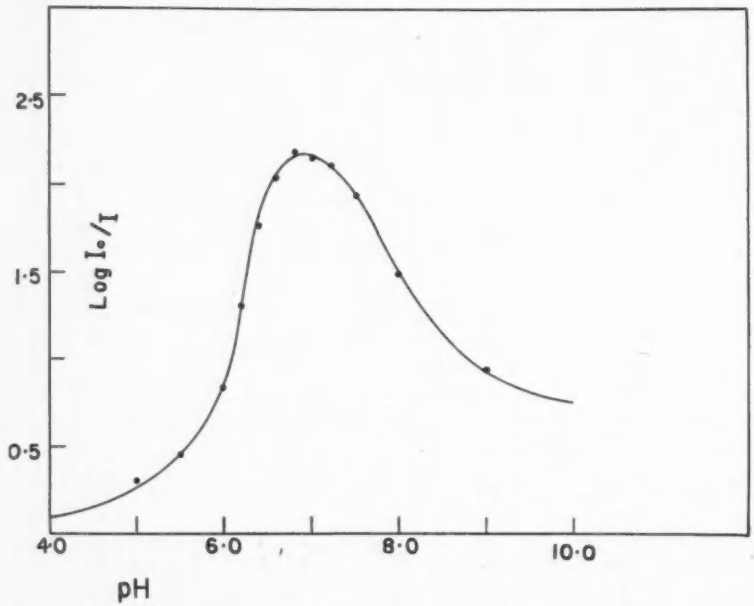


Figure 8. Effect of pH on the activity of herring lipoxidase. (2 mg. linoleic acid substrate and 0.01 mg. of enzyme.)

Effect of Temperature on the Enzyme Activity

A series of trials was made at each of several temperatures, using equal quantities of enzyme together with an optimal amount of activator. The relative activities were measured as the optical density and the results are shown in Figure 9. It must be pointed out that the rapid decrease in reaction rate above 20°C. does not represent a thermal destruction of the enzyme, for during its pre-

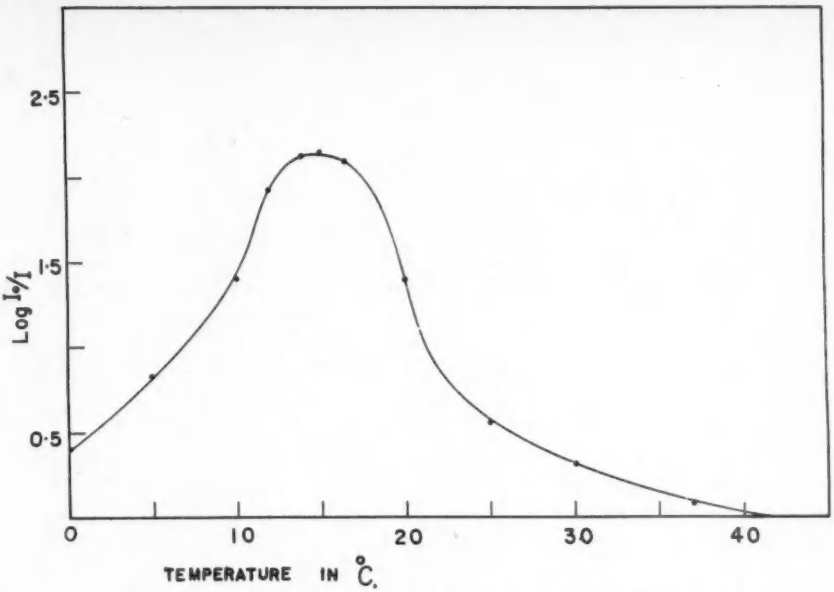


FIGURE 9. Effect of temperature on herring lipoxidase activity. (2 mg. linoleic acid substrate and 0.01 mg. of enzyme.)

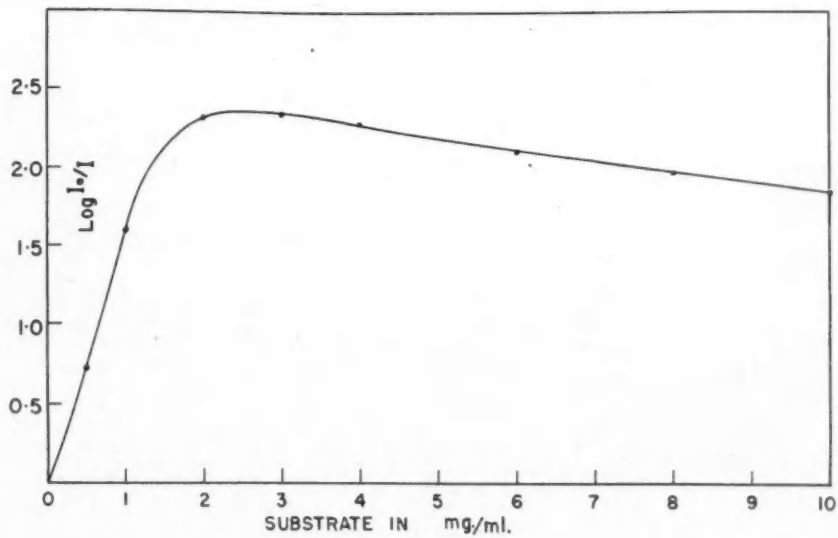


FIGURE 10. Effect of substrate concentration of enzyme activity. (0.01 mg. enzyme.)

paration the enzyme endured a much higher temperature for a longer period of time without much loss in activity. The decrease in activity at higher temperatures might possibly be due to an inactivation of the enzyme in contact with substrate or reaction products.

Effect of Substrate and Enzyme Concentration on the Enzyme Activity

In the presence of an optimal amount of enzyme and activator, the activity is a function of the substrate concentration. Thus in the presence of 0.01 mg. enzyme and 8×10^{-4} gm. of activator (a), the reaction is monomolecular up to a linoleic acid concentration of 2 mg. per ml.; but in solutions containing between 4–7 mg. per ml. of linoleic acid there is little or no influence of substrate concentration on enzyme activity. In other words, the reaction rate is of zero order. At higher concentrations of substrate the activity again decreases. These results are shown in Figure 10.

Similar results were obtained when the concentrations of the substrate and the activator (in optimal amounts) were kept constant and that of the enzyme varied. At concentrations below 0.01 mg. of enzyme per millilitre of 0.200 per cent linoleic acid the activity was directly proportional to the enzyme concentration. If the enzyme concentration was further increased, the reaction rate was not influenced; while with quantities of more than 0.10 mg. of enzyme the reaction rate decreased. These results are shown in Figure 11.

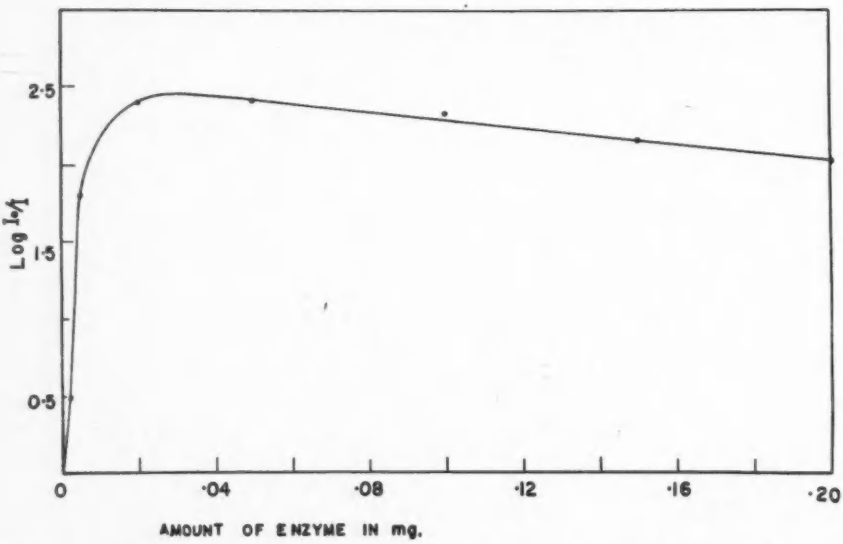


Figure 11. Effect of enzyme concentration on the activity of herring lipoxidase. (2 mg. linoleic acid substrate.)

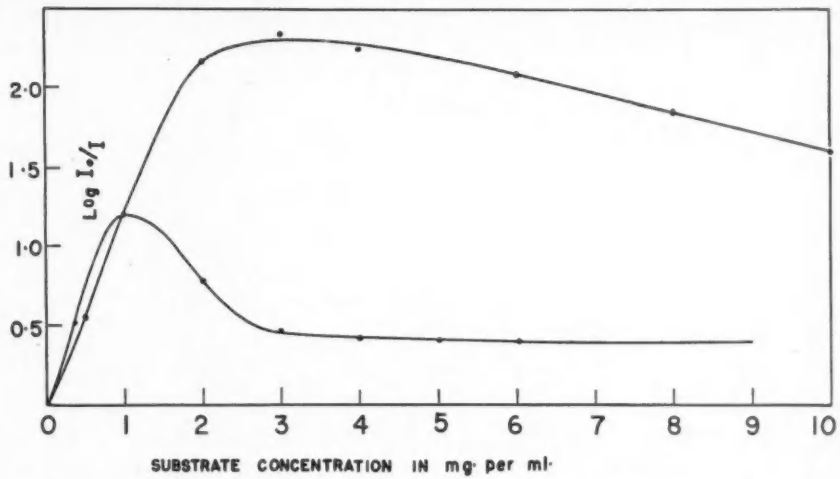


FIGURE 12. Effect of substrate concentration on the activators. The upper curve represents activity at different substrate concentrations in presence of their respective optimal quantity of enzyme and activator (*a*). The lower curve shows the effect of substrate concentrations in presence of an optimal amount of enzyme and activator (*a*) for 1 mg. substrate.

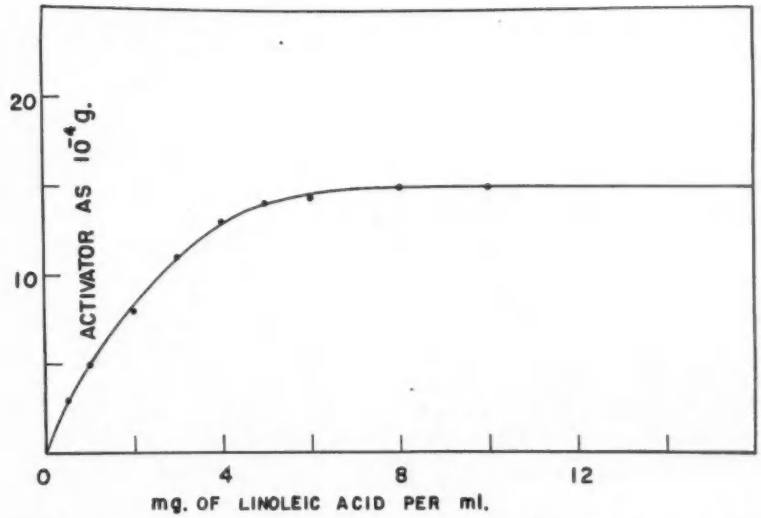


FIGURE 13. Optimal amount of activator at different substrate concentrations. Enzyme concentration constant (0.01 mg.).

Enzyme Activators

The effects of various amounts of both activator (*a*) and activator (*b*) as well as of mammalian haematin, haemaglobin, and cytochrome "c" were studied. The effect was observed by measuring the total optical density produced at 2380 Å under the conditions described previously. Activator (*b*) is roughly 1.6 times as active as activator (*a*) (Table IV). The amount of activator needed to produce maximal peroxide formation in a given time depended upon the quantity of fat present. If activator and the enzyme were both held constant, the maximum peroxide formation occurred in the presence of a definite quantity of fat (Figure 12). Either increase or decrease in the amount of fat resulted in a decrease in the peroxide formed. However, when the amount of activator was increased together with fat, the rate of oxidation increased with substrate concentration. The proportion of activator may, therefore, be increased to compensate for an increase in fat. Large proportions of activator are apparently inhibitory (Figure 13), giving in effect a broad optimal of activator concentration. Furthermore, it is shown that the optimal amount of activator required to produce maximal peroxide formation within a given time was independent of the amount of enzyme present (Figure 14). Relative activities of various activators are shown in Table IV.

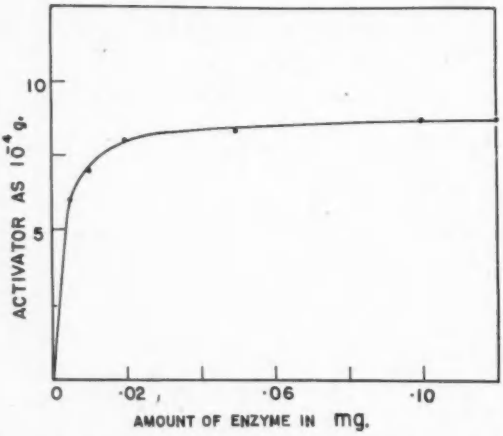


Figure 14. Optimal amount of activator at different enzyme concentrations. Substrate concentration constant (2 mg.).

Enzyme Inhibitors

The activities in presence of inhibitors were determined in the manner described, but using 0.5 ml. of substrate containing 4 mg. linoleic acid per ml. and 0.5 ml. of the appropriate inhibitor solution. Use of the ferric thiocyanate assay method was necessary in cases where ultra-violet absorption of the inhibitor was too high in the blanks.

Effects of some commonly employed enzyme inhibitors are shown in Figure 15. Cyanide inhibited the lipoxidase-catalysed reaction to a pronounced degree

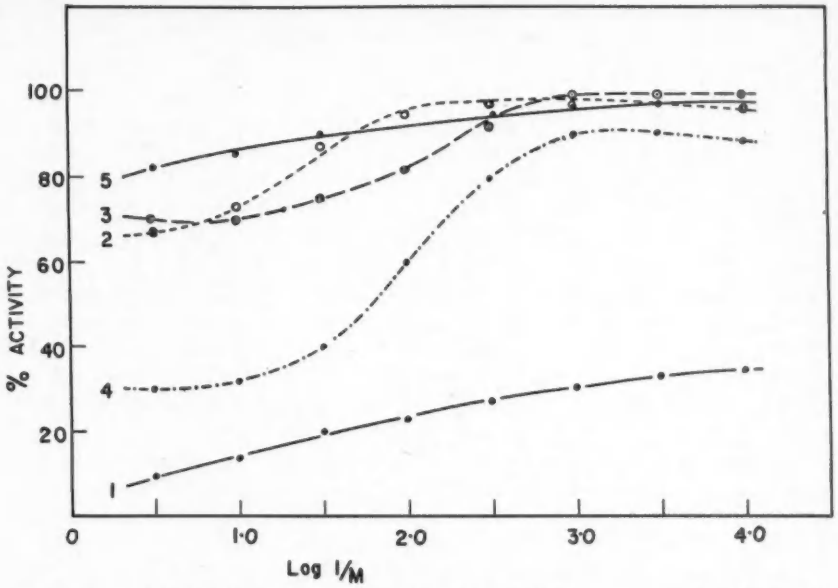


FIGURE 15. Effect of inhibitors on herring lipoxidase activity in presence of activator (*b*), 2 mg. linoleic acid substrate, and 0.01 mg. enzyme. (1) Cyanide, (2) fluoride, (3) arsenite, (4) azide, (5) iodoacetic acid.

at all concentrations of the inhibitor; azide exhibited considerable inhibitory effects only at higher concentration, while fluoride and arsenite were slightly inhibitory at higher concentrations. Iodoacetate exhibited very little or no inhibitory effects at all concentrations. At lower concentrations, however, fluoride, arsenite, and azide caused little or no inhibition.

Irrespective of whether activator (*a*) or (*b*) is present in the enzymic reaction, these effects of the inhibitors were similar in character although they varied slightly in degree. For example, when activator (*a*) was present with 0.5 M cyanide no activity of the enzyme remained; while under similar conditions in presence of activator (*b*) about 10 per cent of the enzymic activity persisted. It is still uncertain whether the reduced activity is due to the inhibition of the enzyme proper or of its activator. But since the enzyme does not contain any heavy metal, while the activators are characterized by the presence of iron in organic combination, it would seem possible that the inhibition by cyanide and azide is due to the inactivation of the activators rather than of the enzyme proper. The failure of iodoacetic acid to inhibit the activity and also the fact that the nitroprusside and other tests for sulphur were negative, rule out the sulphydryl groups as active centres.

DISCUSSION

From the results it is concluded that herring fish contains a proteinaceous, heat-labile enzyme capable of peroxidizing naturally occurring unsaturated fatty

acids, particularly those possessing methylene-interrupted double bonds. This enzyme exhibits optimal activity at 15°C. and at pH 6.9 but fails to catalyse unless some iron-containing, organic, heat-stable activator is present along with it. The enzyme which is here termed "lipoxidase" is characterized by an absence of heavy metals and sulphydryl group as the active centre. The inhibition of the enzymic reaction brought about by cyanide and azides is possibly due to the inactivation of the iron-containing proteinaceous activators, rather than inactivation of the enzyme itself.

It must, however, be emphasized that the accessory substances here termed "activators" do not bring about the activation of the enzyme proper. In other words, the lipoxidase is isolated in an active form, not as a zymogen; because if the enzyme were present as a zymogen and the activators were kinases, the alleged zymogen of lipoxidase would long have been converted to its active form since in the initial stages of purification of the enzyme the activators (or kinases) and the lipoxidase (or zymogen) were in contact with each other for a protracted period.

The activators appear to be conjugated proteins in combination with an iron-containing prosthetic group. This group is rather firmly attached to the protein portion. However, the former can be removed from the latter by treatment with moderately strong alkalis and acids.

The absorption spectrum of the prosthetic group of the activators resembles very closely that of the mammalian haematin. From this evidence and also from the fact that the prosthetic group is an iron-containing non-protein nitrogenous compound, it is believed that the concerned component of the activators is haematin.

The absorption spectrum of activator (*a*) resembles fairly closely that of mammalian haemoglobin. The slight deviations of the above spectrum from that of mammalian haemoglobin might possibly be due to differences in particle size, structure, and composition of the protein fraction of activator (*a*). In all probability, therefore, activator (*a*) is herring haemoglobin. Further evidence of the haemoglobin character of activator (*a*) is provided by the fact that the activity of herring lipoxidase is augmented by mammalian haemoglobin to a degree closely similar to that caused by activator (*a*) (Table IV).

Unlike the absorption spectrum of activator (*a*), that of activator (*b*) does not so closely resemble the absorption spectrum of any known haemochromogen. However, the maximum lies within the range for the cytochromes and is situated closer to the maximum for mammalian cytochrome "c" than to that for cytochrome "b". This phenomenon could be explained on the assumption that the protein fraction of herring cytochrome has a molecular size, composition, and structure different from those of the protein fraction of mammalian cytochrome.

In inhibition experiments with cyanide it was found that even at 0.5 M concentration of the inhibitor about 8 per cent of the activity persisted. It is known that cytochrome "c" is completely inhibited at such a concentration while cytochrome "b" is inhibited up to about 90 per cent. Furthermore, the activity of

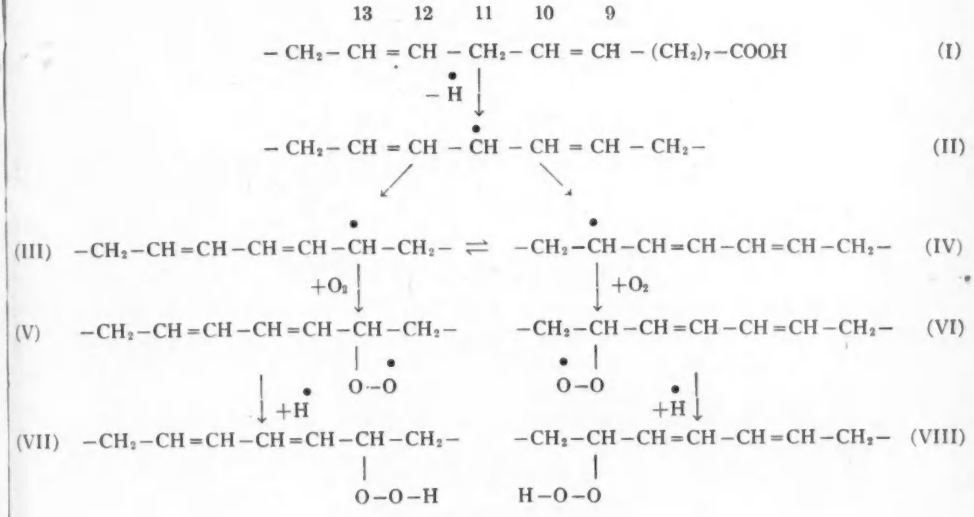
mammalian cytochrome "c" towards the herring lipoxidase-catalysed reaction is about 75 per cent that of activator (*b*) (Table IV).

On the basis of the evidence obtained, it is believed that activator (*b*) is herring cytochrome "c" possibly contaminated with herring cytochrome "b".

Although the prosthetic group alone of the activators exhibits activity, its combination with a protein greatly modifies the degree of activity. It is as yet uncertain what specific role these activators play. But it would seem plausible to assume that due to the presence of the haematin fraction in their molecules, these compounds constitute an oxidation-reduction system.

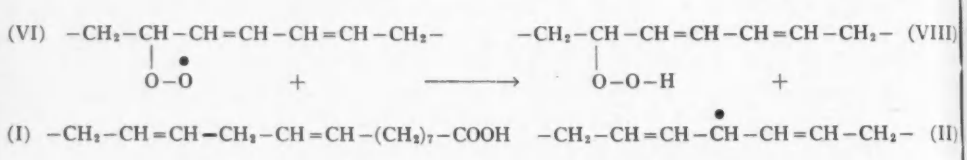
Banks (1949) reported earlier work on the catalytic system responsible for the increased oxidation of the fat of herring during cold storage. Cytochrome "c" was found to be the most, and haemoglobin the least, active of the natural pigments, with haematin being no more active than methaemoglobin. Linoleic acid was employed as substrate. Small amounts of iron and copper had very little effect on the reaction, and it appeared that the catalytic oxidation of the fat of cold-stored herring is due at least in part to the pigments of the flesh and blood. Preliminary spectrographic examination of the lateral band of pigmented muscle of herring indicated it to be particularly rich in cytochrome components.

In accordance with evidence resulting from investigations by Bergström (1945), Holman (1946) and other workers in the field, as well as Bergström and Holman (1948), and Holman (1947), the mechanism of reaction involved in the oxidation of linoleic acid catalysed by a lipoxidase might be as follows:



(• indicates activation)

The oxidation of linoleic acid, once started by lipoxidase, could continue by means of a chain reaction. For example, the radicals V or VI could accept a hydrogen atom from linoleic acid:



giving rise to another radical II which would then go through the same cycle. Lipoxidase may thus have its function in initiating the chain reactions by removal of hydrogen from the linoleic acid methylene group.

Other authorities (Swern, Scanlan and Knight, 1948) have suggested that the oxygen addition may occur at stage II, followed by re-arrangement of the unsaturated double bond, instead of the double-bond re-arrangement preceding the addition of oxygen at stage III as in the scheme indicated above. The end result is the same in either case.

The activators possibly accept an atom of hydrogen from linoleic acid at stage I and donate the hydrogen atom back at stage V or VI.

In concluding the above discussion, attention may be drawn to the practical use of antioxidants to combat in oily fish products such as frozen herring and salmon the tendency of their oils to become rancid during prolonged cold storage. The present investigation has been closely linked with investigations of antioxidants at the Pacific Fisheries Experimental Station. Tarr (1947, 1948) reported the comparative values of a number of antioxidants in delaying the onset of rancidity in indigenous fats of fish. Tarr and Cooke (1949) and Khan (1948), studied the effects of certain cyclic and acyclic carbonyl enediols in retarding rancidity when fish fillets and steaks are dipped in solutions of these compounds prior to freezing, as well as when these compounds are incorporated in the glaze. A recent revision of the Canadian Pure Food and Drugs Act recognizes the use of ascorbic acid as an antioxidant for fish flesh.

SUMMARY

1. Three different methods of estimating lipoxidase activity in herring flesh were investigated. The ultra-violet light adsorption method was adopted with certain modifications.

2. Previous reports that lipoxidase in herring is concentrated in the dark muscle along the lateral lines and that the flesh exhibits little or no lipoxidase activity were confirmed. Samples of the dark muscle from different lots of herring were found to possess different lipoxidase activity.

3. From the dark muscle was isolated, by different physical and chemical methods of fractionation and finally by electrophoresis, an enzyme over two thousand times as active as the raw material. Attempts to crystallize the enzyme failed.

4. Herring "lipoxidase" is a proteinaceous, heat-labile enzyme capable of peroxidizing naturally occurring unsaturated fatty acids and fatty acid esters, particularly those possessing methylene-interrupted double bonds. It exhibits optimal activity at 15°C. and at pH 6.9 but fails to catalyse unless some iron-containing, organic, heat-stable activator is present. The enzyme is characterized by an absence of heavy metals and sulphydryl group as the active centre.

5. Among the activators for herring lipoxidase, two compounds were isolated from herring flesh. These compounds are conjugated proteins combined with an iron-containing nitrogenous prosthetic group. This group, which exhibits maximal absorption near 4480 Å and at 5610 is believed to be haematin. Activator (*a*) exhibits two absorption maxima, one at 5450 and the other at 5630 Å, while activator (*b*) shows maximal absorption near 5560 Å. On the basis of evidence obtained, activator (*a*) is believed to be herring haemoglobin. Activator (*b*) is considered to be herring cytochrome "c" possibly contaminated with cytochrome "b".

6. There is an optimal concentration of enzyme, substrate, and also of the activators, for maximal enzyme activity. The presence of activators appears to change the kinetics of the oxidation of the substrate.

7. The inhibition of the enzymic reaction brought about by cyanide and azide is possibly due to the inactivation of the iron-containing proteinaceous activators rather than inactivation of the enzyme itself.

ACKNOWLEDGMENTS

Financial assistance from the Government of India, later from the Government of Pakistan, also from the University of British Columbia and the Fisheries Research Board of Canada during the latter part of this investigation, is gratefully acknowledged.

Appreciation is expressed for the counsel and guidance of Dr. N. M. Carter, Director of the Pacific Fisheries Experimental Station of the Fisheries Research Board of Canada, of Dr. W. A. Clemens, Head of the Department of Zoology, University of British Columbia, and of Dr. W. S. Hoar, Professor of Zoology and Fisheries in that Department, who made arrangements for facilities for carrying out the investigation and under whose supervision it was performed.

Dr. Hoar kindly provided certain laboratory facilities at the University, and Dr. Otto Blüh of the Department of Physics kindly made available the use of the Tiselius electrophoretic apparatus and the Beckman spectrophotometer at the Biophysics Laboratories. To them, to Dr. S. H. Zbarsky, Professor of Biochemistry at the University, and to Dr. H. L. A. Tarr of the Pacific Fisheries Experimental Station, acknowledgment of their assistance and helpful suggestions is made.

REFERENCES

Balls, A. K., B. Axelrod and M. W. Kies. *J. Biol. Chem.*, **149**, 491–504, 1943.
Banks, A. *J. Soc. Chem. Ind.*, **56**, 13T–15T, 1937.
J. Soc. Chem. Ind., **63**, 8–13, 1944.
Rept. of the Food Invest. Bd. (D.S.I.R.) for 1939, 49–50, 1949.

BERGSTRÖM, S. *Arkiv. Kemi. Mineral. Geol.*, **21A**, 1–18, 1945.
BERGSTRÖM, S., AND R. T. HOLMAN. *Nature*, **161**, 55, 1948.
BLACK, H. C. *U.S. Quartermaster Corps Manual, QMC* **17–7**, 35–39, 1945.
BROCKLESBY, H. N. AND N. I. ROGERS. *Fish Res. Bd. Can., Prog. Rep. Pac.*, **50**, 4–8, 1941
CRAIG, F. N. *J. Biol. Chem.*, **114**, 727–746, 1936.
GREENWOOD, D. A., J. E. STRITER AND H. R. KRAYBILL. *Proc. Inst. Food Tech.*, 58–71, 1945.
HILLS, G. L. *J. Council Sci. Ind. Research*, **19**, 1, 1946.
HILLS, G. L., AND J. CONOCHIE. *J. Council Sci. Ind. Research*, **18**, 355–365, 1945.
HOLMAN, R. T. *Arch. Biochem.*, **10**, 519–529, 1946.
Arch. Biochem., **15**, 403–413, 1947.
Advances in enzymology, Vol. 8, 425–457. New York and London: Interscience Publishers, Inc., 1948.
HOLMAN, R. T., AND G. O. BURR. *Arch. Biochem.*, **7**, 47–54, 1945.
HOLMAN, R. T., AND O. C. ELMER. *J. Am. Oil Chemists' Soc.*, **24**, 127–129, 1947.
HOVE, E. L. *Science*, 98, 433–434, 1943.
HUMMEL, J. P., AND H. A. MATTIL. *Proc. Soc. Exptl. Biol. Med.*, **55**, 31–33, 1944.
KHAN, M. R. Dissertation for the degree of Master of Arts, University of British Columbia, 1948.
KOKATNUR, V. R., AND M. JELLING. *J. Am. Chem. Soc.*, **63**, 1432–1433, 1941.
LANG, K. *J. Physiol. Chem.*, **261**, 240–248, 1939.
LEA, C. H. *J. Soc. Chem. Ind.*, **50**, 215T–220T and 343T–349T, 1931.
J. Soc. Chem. Ind., **52**, 57T–63T, 1933.
J. Soc. Chem. Ind., **56**, 376T–380T, 1937.
Rancidity in edible fats. New York: Chemical Publishing Co. Inc., 1939.
LEHNINGER, A. L. *J. Biol. Chem.*, **161**, 413–414 and 437–451, 1945.
LEHNINGER, A. L., AND E. P. KENNEDY. *J. Biol. Chem.*, **173**, 753–771, 1948.
MATTIL, H. A. *Oil and Soap*, **18**, 73–76, 1941.
MUNOZ, J. M., AND L. F. LELOIR. *J. Biol. Chem.*, **147**, 355–362, 1943.
OSER, B. L. *Food Ind.*, **18**, 1683–1685 and 1822–1828, 1946.
REISER, R. *J. Am. Chemists' Soc.*, **26**, 116–120, 1949.
REISER, R., AND G. S. FRAPS. *J. Assoc. Offic. Agr. Chemists*, **26**, 186–194, 1943.
SÜLLMAN, H. *Helv. Chim. Acta*, **24**, 1360–1380, 1941.
Private communication (1947) referred to in Holman, *Advances in Enzymology*, Vol. 8, 443. New York and London: Interscience Publishers, Inc., 1948.
SUMNER, J. B. *J. Chem. Education*, **19**, 70, 1942.
SUMNER, J. B., AND A. L. DOUNCE. *Enzymologia*, **7**, 130–132, 1939.
SUMNER, J. B., AND G. N. SMITH. *Arch. Biochem.*, **14**, 87–92, 1947.
SUMNER, J. B., AND G. F. SOMERS. Laboratory experiments in biological chemistry, p. 138. New York: Academic Press, 1944.
SUMNER, J. B., AND R. J. SUMNER. *J. Biol. Chem.*, **134**, 531–533, 1940.
SUMNER, R. J., *J. Biol. Chem.*, **146**, 211–213, 1942a.
J. Biol. Chem., **146**, 215–218, 1942b.
Ind. Eng. Chem., Anal. Ed., **15**, 14–15, 1943.
SWERN, D., J. T. SCANLAN AND H. B. KNIGHT. *J. Am. Oil Chemists' Soc.*, **25**, 193–200, 1948.
TARR, H. L. A. *J. Fish. Res. Bd. Can.*, **7**, 137–154, 1947.
J. Fish. Res. Bd. Can., **7**, 248–253, 1948.
TARR, H. L. A., AND N. E. COOKE. *J. Fish. Res. Bd. Can.*, **7**, 522–527, 1949.
TAUBER, H. *J. Am. Chem. Soc.*, **62**, 2251, 1940.
THEORELL, H., S. BERGSTROM AND A. AKESON. *Pharm. Acta Helv.*, **21**, 318–324, 1946.
WATTS, B. M., AND D. PENG. *J. Biol. Chem.*, **170**, 441–453, 1947.
YOUNG, C. A., R. R. VOGT AND J. A. NIEUWLAND. *Ind. Eng. Chem., Anal. Ed.*, **8**, 198–199, 1936.